Award Number: W81XWH-11-1-0580

TITLE: Targeting Microglia to Prevent Post-Traumatic Epilepsy

PRINCIPAL INVESTIGATOR: Daniel S. Barth

CONTRACTING ORGANIZATION: University of Colorado, Boulder, CO 80309

REPORT DATE: July 2013

TYPE OF REPORT: Annual report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE	*Form Approved* *OMB No. 0704-0188*

1. REPORT DATE July 2013	**2. REPORT TYPE** Annual	**3. DATES COVERED** 1 July 2012 – 30 June 2013
4. TITLE AND SUBTITLE Targeting Microglia to Prevent Post-traumatic Epilepsy		**5a. CONTRACT NUMBER** W81XWH-11-1-0580
		5b. GRANT NUMBER W81XWH-11-1-0580
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Daniel S. Barth		**5d. PROJECT NUMBER**
		5e. TASK NUMBER
E-Mail:		**5f. WORK UNIT NUMBER**
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Colorado, Boulder, CO 80309		**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		**10. SPONSOR/MONITOR'S ACRONYM(S)**
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose of this research project is to explore anti-epileptogenic strategies in and animal model of post-traumatic epilepsy (PTE) using lateral fluid percussion injury (LFPI). Our focus is on attenuating damaging effects of hyperexcitability in the brain induced by inflammation resulting from glial cell immune responses to trauma. We are exploring two drugs, MN166 and SLC022, that are known to suppress post-traumatic glial activation and thus inflammation to evaluate their effectiveness in preventing epileptogenesis in the LFPI model of PTE. In this first project year we have developed a high-speed video/EEG recording and analysis system for rapid quantification of chronically recorded epileptiform activity in multiple (24-32) subjects. With this system we have become expert in identifying epileptiform versus normal video/EEG activity in the rodent and have discovered an important source of artifact currently being interpreted in other published reports as seizure activity. We have developed a pilocarpine model of temporal lobe epilepsy to explore the effectiveness of glial cell (neuroimmune) attenuation in preventing or limiting epileptogenesis (development of epilepsy) in this rapidly developing model. We are making changes in our LFPI model to produce earlier developing signs of epilepsy, increasing the probability of succeeding in our long-term study of epileptogenesis following traumatic brain injury. Finally, we discovered and published results concerning development of post-traumatic anxiety in our brain injured animals that we could effectively prevent with peri-injury administration of glial attenuating drug, MN-166, the same drug to be used in our studies concerning prevention of epileptogenesis following traumatic brain injury.

15. SUBJECT TERMS
Post-traumatic epilepsy, traumatic brain injury, neuroinflammation, neuroimmune

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	125	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS:

INTRODUCTION:

Post-traumatic epilepsy (PTE) is a common result of traumatic closed head injury. The development of epilepsy (epileptogenesis) can take many months to several years before the appearance of behavioral seizures. Compared to other forms of epilepsy, PTE is particularly resistant to antiseizure medication once it has developed and there are currently no therapeutic interventions to prevent or attenuate epileptogenesis. The purpose of this research project is to explore anti-epileptogenic strategies in and animal model of PTE using lateral fluid percussion injury (LFPI). Our focus is on attenuating damaging effects of hyperexcitability in the brain induced by inflammation resulting from glial cell immune responses to trauma. We are exploring two drugs, MN166 and SLC022, that are known to suppress post-traumatic glial activation and thus inflammation to evaluate their effectiveness in preventing epileptogenesis in the LFPI model of PTE. If successful, our results could have accelerated impact on translation to preventing PTE in war fighters since one of these drugs (MN166) has already been approved by the FDA and is in clinical trials for human neuropathic pain studies.

BODY:

YEAR 1

There were two objectives for year one of this project. The first was to construct a custom video/EEG acquisition/analysis system. The second was to record sensory evoked potentials and spontaneous EEG from acutely anesthetized animals receiving LPS applied directly to the cortical surface to evaluate the effectiveness of MN166 in reducing microglial TLR-4-mediated hyperexcitability.

Progress on objective 1: The custom video/EEG acquisition and analysis system is complete and fully functional (**Fig. 1**). This consists of two racks with 16 recording chambers each. Each recording chamber was custom made and consists of a 12" diameter and 24" high plastic cylinder equipped with a 7 channel electrode harness that is attached on one end to chronic screw electrodes on each rat and on the other end to a slip-ring swivel connector, permitting recording with unimpeded movement. Each recording chamber is also equipped

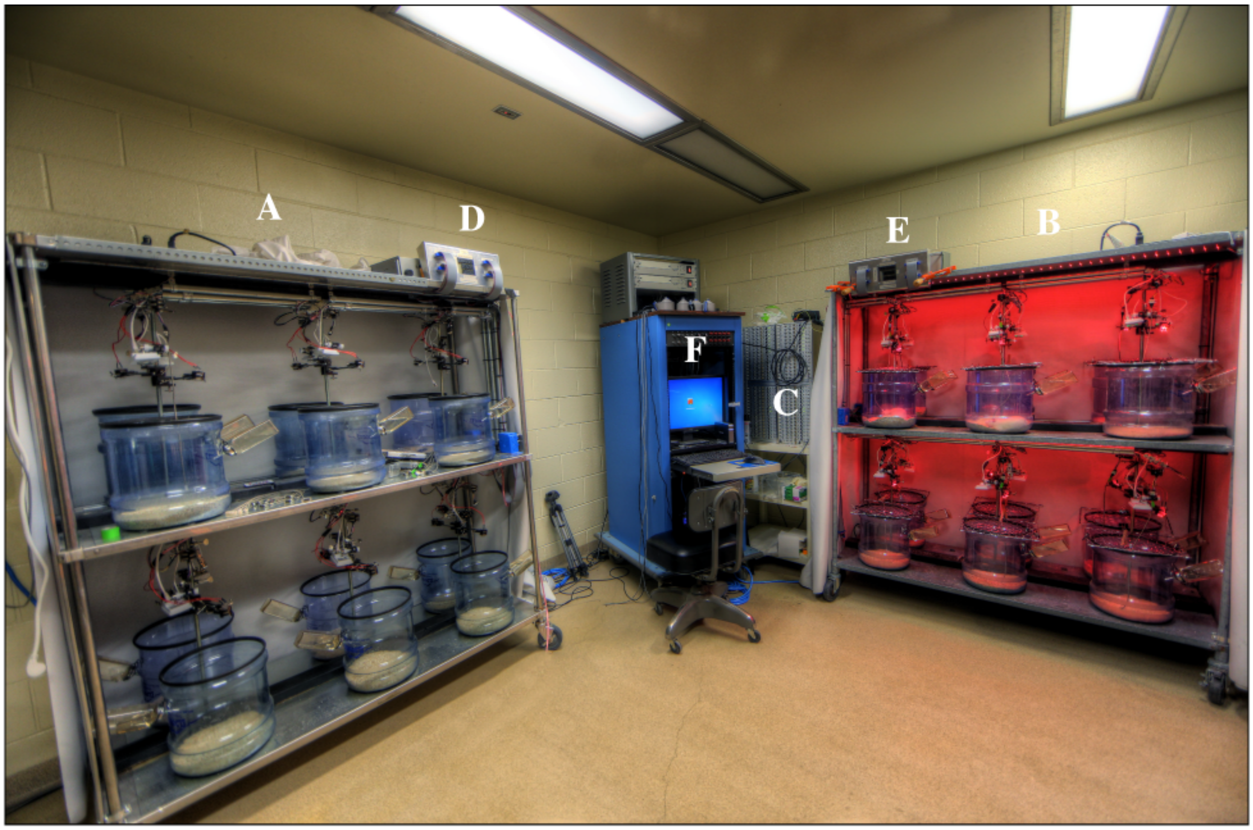


Fig. 1. Wide angle view of chronic recording rigs used for video/EEG of 24 (max 32) rats simultaneously. A&B) Dual level platforms holding 6 chambers per level. Each chamber consists of a containment vessel, swivel electrode harness and video camera (shown in higher resolution in next figure). All cameras are IP survailence cameras multiplexed through high speed internet switches on the top of each rig. C) 64 channel EEG amplifier used for our first recordings. D&E) These have now been replaced by 2 custom made 64 channel EEG amps of much smaller dimension. F) Rack cabinet with power supplies and DAQ computer.

with a dedicated surveillance camera (Axis M1011) for recording video. We chose cameras that are designed for internet protocol (IP) recording because they can easily be multiplexed through a wired or wireless router and use compression (H264) to reduce bandwidth (**Fig. 2**), which is critically important for chronic video/EEG recording and analysis. Each chamber is also equipped with DC (light emitting diode) lighting for day (white) and night (red) video recording without disturbing sleep cycles. Two compact 64-channel EEG amplifier systems (designed by the P.I.) were also constructed (one for each rack) to buffer signals before digitization and computer storage. The digital acquisition software was written by the P.I. in Visual Basic and provides a flexible means of logging EEG and simultaneous video for each rat in date/time registered folders. The need to log video along with EEG posed a particular challenge due to the bandwidth of video and the need to precisely time-lock the signal to each rat's EEG. This problem was solved in part through using IP cameras as noted above. The final solution to the problem was to use computers capable very large RAM storage so 30 minute trials of temporally contiguous EEG could be sampled without interrupt from all rats while spooling video to disk and finally writing the EEG at the end of each trial while the cameras are paused. This data collection hardware/software was finished early and has been fully functional for several months, permitting us to get a head start on chronic video/EEG recording.

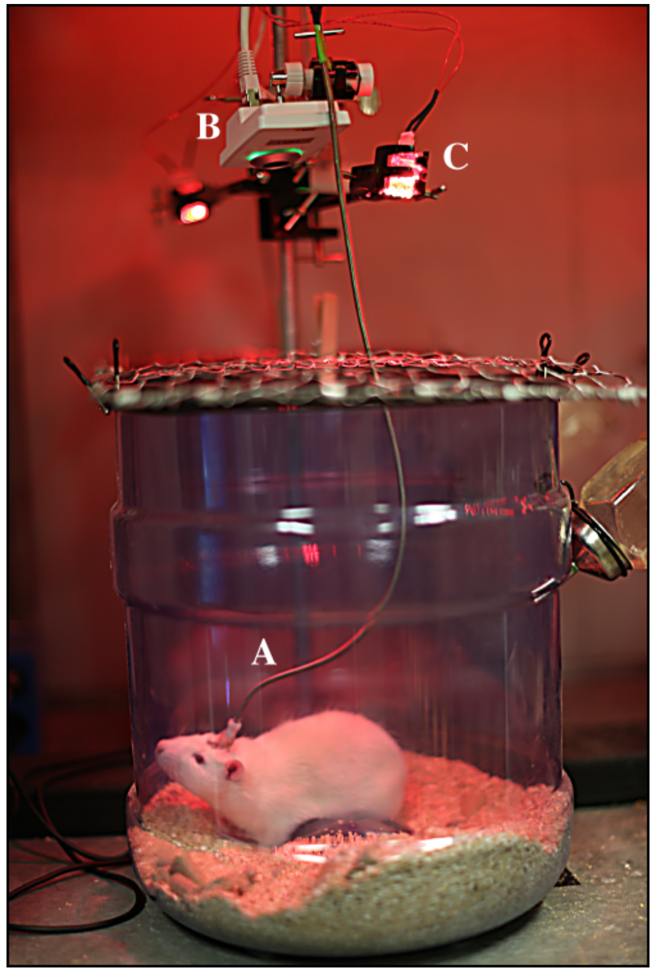


Fig. 2 Close up view of single recording chamber. A) EEG channel plug for rat head mount attached to flexible electrode harness and slip-ring commutator. B) Survailence camera (1 per rat) providing highly compressed H264 images to limit band-width demands. C) High intensity red LEDs for night recording.

Our analysis hardware and software for the video/EEG data has also been completed and is fully functional. This turned out to be the most challenging part of the project since there is presently no commercially available software that permits extremely rapid inspection of these enormous data sets recorded 24/7 from large numbers of animals. The hardware finally chosen consists of PCs designed for gaming, providing very fast numerical and video processing at moderate cost. Video data is displayed on two high-resolution monitors mounted in tandem, permitting visual inspection of 30 min of EEG in a single page. All data analysis software was written by the P.I. in the MatLab environment and, to our knowledge, exceeds anything commercially or privately available for exploring these large data sets. From the P.I.'s previous experience of the pitfalls of automated analysis of epileptiform EEG data, the design principal of the present software was to permit initial rapid <u>visual</u> inspection of all data, and to use automated analysis only for subsequent quantification of suspected epileptiform events. As noted above, EEG data for a given rat is rapidly presented in 30 min blocks. The operator can rapidly zoom in on suspected epileptiform events and precisely mark their latency with a mouse click for event logging and subsequent quantification. Zooming also defines a time window within which clicking on a trace plays the video clip associated with that window for verification of seizures. Thus, unlike existing review software, our program permits quasi-random access to the data accompanied by user defined video review. This software has now been in extensive use and meets our design goal of reviewing a full one-day data set in 5-10 min. Since we finished the data collection and analysis system ahead of schedule, we have had several months to begin looking at spontaneous recordings from normal and brain damaged rats (noted below). This has prompted two additions to the software for quantifying results. The first was designed for automated epileptic spike detection. Typical spike detection programs commercially available are based on attempting to use universal spike descriptors (i.e. amplitude, rise-time, etc.) to separate spikes from noise. These approaches, while easier to implement, suffer from numerous false positives and noise. The approach we took instead was to take advantage of our ability to rapidly visually identify sub-sets of spikes for each rat <u>individually</u>, and from these make a rat specific average spike template that is sequentially matched to the actual data using a covariance measure that is thresholded to separate signal from noise. This approach is quite accurate, and with our fast processors, can count spikes over many days of data in under an hour. We have also added a feature to the software that employs a touch screen to permit rapid but manual identification and quantification of more prolonged epileptiform events such as seizures and seizure like artifacts (noted below) for subsequent video verification. The speed of this quantification is achieved by using foot pedals to signal the event type and a wand on the touch screen to mark event time and duration. A brief video demonstration of this analysis software was provided to our Science Officer, Dr. Jordan D. Irvin, and is downloadable at http://dl.dropbox.com/u/11873936/SoftwareDemo1.wmv. We will also be presenting this work at The 2012 Military Health System Research Symposium held 13-16 August 2012 in Fort Lauderdale, Florida. The abstract for this presentation is included in Appendix. We feel our video/EEG data collection/analysis system should serve not just our own research but is sufficiently unique, fast, and inexpensive to be useful for emergency and post-emergency monitoring of soldiers suffering traumatic brain injury in the battlefield.

Progress on objective 2: The second objective of this first year project was to determine the efficacy of attenuating glial cell activation (using MN-166 and SLC022) in decreasing acute hyperexcitability of the brain induced by lipopolysacharride (LPS) applied directly to the cerebral cortex of anesthetized rats. Upon initial investigation we realized that anesthesia was having an unacceptable and variable influence on cortical excitability induced by LPS. Thus, while we could suppress excitability through glial attenuation, these results were confounded by additional suppressive anesthesia effects. Particularly troublesome was the fact that the effect of various anesthesia regimes we tried (ketamine/xylazine, xylazine alone, isoflurane, urethane) had highly variable effects in both increasing or decreasing the response to LPS independent of glial modulating treatment. With permission of our Scientific Officer, we decided to abandon this study in order to devote our time instead to accelerating work on unanesthetized animals. This turned out to be a good decision for several reasons:

1) We got a head start on examining chronic video/EEG recording from rats with and without lateral fluid percussion injury (LFPI). We were able to examine these initial recordings with unprecedented accuracy since our software relies on visual as opposed to automatic review. It immediately became apparent to us that both our control and LFPI rats displayed a repertoire of EEG patterns associated with chewing, grooming etc., which

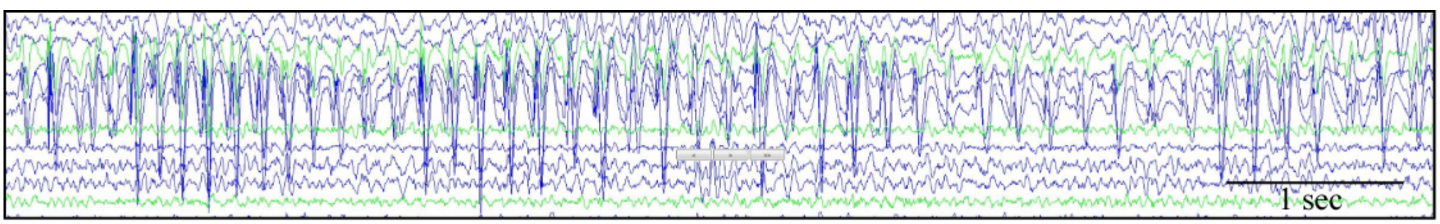


Fig. 3. Typical theta activity recorded from rat sensorimotor cortex during "bruxing" and "eye boggling".

are normal artifacts that we are now expert in recognizing. However, an unexpected finding was that both the LFPI and the control rats displayed pronounced runs of theta activity (Fig. 3). This drew our attention because the theta activity we recorded was similar in frequency, amplitude, and duration to Epileptiform Electrographic Events (EEE) previously associated exclusively with LFPI[1,2]. By examining restrained animals with high resolution video and simultaneous EEG, it became apparent that the theta was not epileptiform but was instead due to "bruxing", also referred to as "vacuous chewing"[3,4], and "eye boggling", both activities that rats perform normally to dull the front incisors as well as when they are under stress. Please see video clip at http://dl.dropbox.com/u/11873936/BruxingBoggling.wmv displaying such behavior in relation to the EEG shown in Figure 3. While this discovery sounds trivial, it is actually at the center of a very recent controversy concerning what can be safely considered to be post-traumatic epileptiform activity in LFPI rats (see [2] and[5] for point/counter-point).

2) In reviewing our initial chronic recordings we realized that we needed more experience discerning normal from epileptiform activity. To this end we received approval to conduct a pilot study using a pilocarpine model of temporal lobe epilepsy. This model involves injecting animals with lithium followed by pilocarpine (a muscarinic receptor agonist) which induces acute status epilepticus for several hours[6]. Status is followed by a "silent period" of several weeks where epileptiform spikes may be recorded, and then the appearance of regular

temporal lobe seizures. We began an initial study in 8 rats using this model and have just started to see seizures at the 4-week time-point following status. Thus, this brief study has served its purpose of familiarizing us with chronic video/EEG recording and analysis of spikes and seizures. However, having succeeded with this model, it provides us with an ideal opportunity to test the effectiveness of glial attenuation in preventing epileptogenesis presumed to occur during the one month silent period before chronic seizures occur. This study in underway and should provide us with valuable insights concerning prevention of epileptogenesis in this more rapidly developing model before proceeding with LFPI animals and a much prolonged (many months) silent period.

3) Finally, our early start on chronic recording led to an unexpected serendipitous finding concerning post-traumatic anxiety. In piloting LFPI rats, we noticed that brain damaged animals displayed behaviors suggesting increased anxiety when placed in the recording chamber. We pursued this by performing an experiment using a controlled stressor (foot shock) and measuring freezing behavior (the rats natural defensive behavior to danger). Indeed, our LFPI animals showed a reliable over-reaction to stress when compared to controls, suggesting an animal model of post-traumatic anxiety. Most important, we found that glial attenuation with peri-injury administration of MN-166 completely prevented development of post-traumatic anxiety. While not directly related to post-traumatic epilepsy, we believe the enhanced post-traumatic anxiety is reflective of increased excitability of limbic structures due to injury-induced neuroinflammation. In this way, our serendipitous discovery holds promise for our epilepsy studies. This work is now published[7] and the manuscript is included in Appendix.

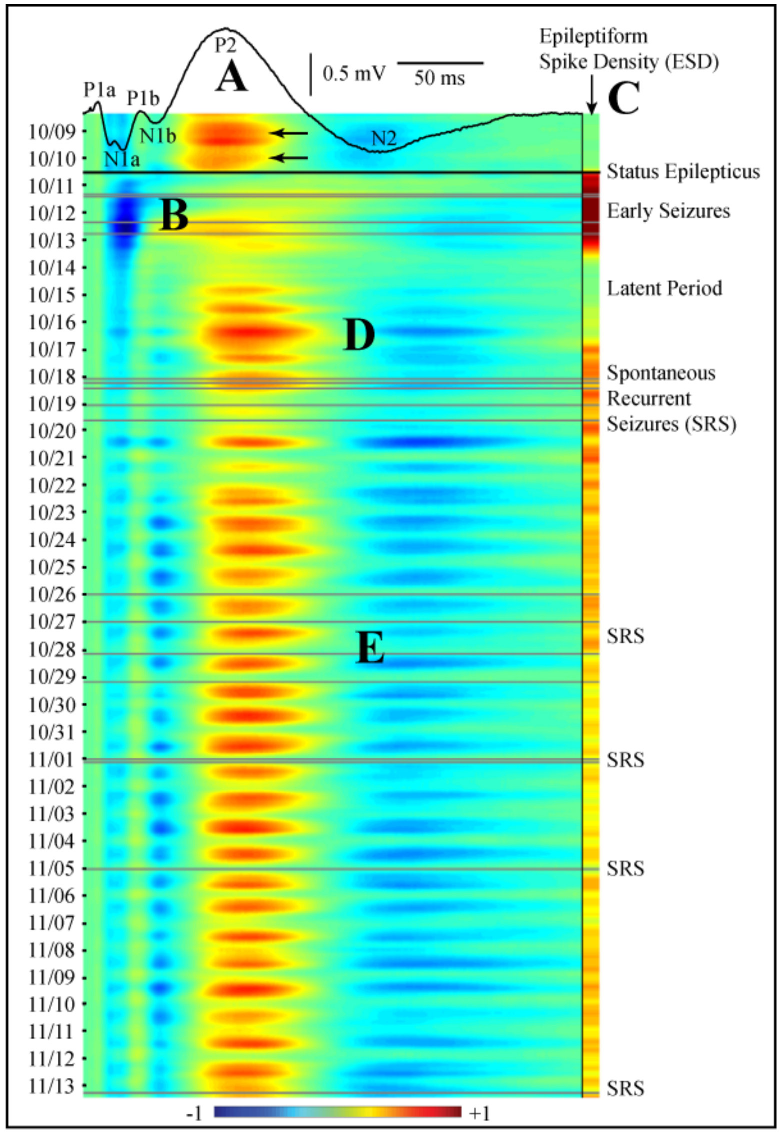


Fig. 4. Auditory evoked potentials (AEP) and epileptiform spike density (ESD) recorded from auditory cortex. A) AEP waveform before status epilepticus (SE). B) Early seziures before epileptogenesis. C) ESD. D) The epileptogenic "silent" or "latent" period. E) End of the latent period and appearance of spontaneous recurrent seizures (SRS).

YEAR 2

There were three objectives for year two of this project. The first was to test the efficacy of MN166 in reducing microglial TLR4-mediated hyper-excitability, epileptiform spiking, and seizures in the pilocarpine model of temporal lobe epilepsy. The second was to test more rostral fluid percussion injury over motor cortex with increased impact pressures for its effectiveness in producing short term (1-2 month) epileptiform spiking. The third was to begin testing the efficacy of MN166 and SLC022 as anti-epileptogenic compounds in PTE induced by lateral fluid percussion injury (LFPI), beginning in year two and continuing through year three.

Progress on objective 1: Success with the pilocarpine model has exceeded what we initially planned. An unexpected discovery was that by recording auditory evoked potentials (AEP) every 30 minutes 24/7, we could develop a reliable (and new) biomarker that sensitively indexes excitability changes during epileptogenesis (previously regarded as the "silent" or "latent" period where changes in the brain could not be monitored due to a lack of seizures). The shape and amplitude of the AEP waveform undergoes dramatic and stereotyped changes compared to pre-pilo baseline following status epilepticus (SE), providing a window on changes and treatment efficacy prior to appearance of the first spontaneous recurrent seizure (SRS). AEPs recorded at 30 minute intervals for approximately one month following SE (from a screw electrode directly above auditory cortex) are shown in Figure 4. The archetypal AEP waveform begins with early fast waves reflecting intra-laminar cortical excitation (Fig. 4A; P1a-N1b) followed by inhibitory slow waves (Fig. 4A; P2-N2). The amplitudes of these components remain stable over the 2-day pre-SE baseline, with a detectable circadian fluctuation of the P2 amplitude (Fig. 4A; arrows). AEP morphology is grossly altered by SE, resulting in a prominent increase in the N1a amplitude (Fig. 4B; 10/11 – 10/13) and attenuation of the P2-N2 slow wave, suggesting decreased inhibition during SE and early seizures. Epileptiform spike density (ESD; Fig. 4C) is maximum during this same period. Both the AEP and ESD are suppressed during the earliest part of the latent period but begin to increase several days before the first SRS (Fig. 4D; 10/18). For weeks following the first SRS, spike density is variable but reveals no distinct temporal pattern and shows no temporal relationship to the occurrence of subsequent SRS. By contrast, the AEP amplitude fluctuation evolves into a more prominent circadian rhythm, particularly noticeable in the inhibitory slow waves, which are regularly suppressed for several hours in the early morning. SRS, when they do occur, are time-locked to these periods when inhibition is presumably minimal (Fig. 4E; 10/19-20, 10/26-29, 11/01, 11/05, 11/13). We have performed similar recordings in 10 rats so far and have found very similar AEP changes in both auditory cortex and hippocampus (which responds to auditory stimuli). We have logged changes in the AEP out to 3 months after SE. We consistently find progressive changes before the first SRS (allowing us to monitor excitability changes and thus epileptogenesis during the latent period) as well as change after the first SRS, demonstrating continued epileptogenesis during subsequent seizures. This later point is particularly important since it is currently a matter of some debate whether epileptogenesis is essentially complete by the first SRS or continues, warranting continued anti-epileptogenic treatment well into the seizure period. Our data strongly suggest the later conclusion.

We are now wrapping up our work with biomarker analysis in the pilocarpine model and looking at the effect of glial cell suppressant MN166 following SE on epileptogenesis (indexed by seizures as well as AEP and ESD changes). What we see so far is promising. Two groups of rats are under study, receiving daily injections of MN166 (10 mg/kg in corn oil; N=8) or vehicle (N=7) beginning at the termination of SE and continuing for up to 30 days. 70% (5 out of 7) of the vehicle treated rats have developed chronic seizures with the average latency from SE to the first seizure of 15.5 days (Fig. 5; red traces). Both the success rate and duration of the latent period are consistent with the published literature using this model. In contrast, only 12% (1 out of 8) rats receiving MN166 have had seizures (Fig. 5; black trace). Even with this low N and short duration of recording, the seizure rates in vehicle rats are significantly greater ($p=0.045$) than those treated with MN166 post-SE. These results suggest that anti-inflammatory treatment may at least have an anti-seizure effect. However, we are encouraged that the 3 treated rats so far that we have had the opportunity to monitor out to 40-50 days post-SE (Fig. 5; oval) continue to show no seizures. These rats therefore have remained seizure free for 10-20 days after MN166 was stopped, approaching the 15.5-day latency period for control rats, suggesting that epileptogenesis may have been attenuated. While we have not yet quantified AEP or ESD in these rats, it should be noted that all of the rats (including the MN166 treated one) that have shown seizures also display prominent epileptiform spiking whereas this has not been seen in any of the seizure free rats. Our work on Objective 1 with the pilocarpine model has resulted in an R01 grant proposal to the NIH on anti-epileptogenesis in this model (see Appendix), a preproposal to the CDMRP to further develop our biomarkers for epileptogenesis with this model using optogenetic stimulation of hippocampus and cortex (see Appendix), and an abstract submission to the upcoming Society for Neuroscience meeting (see Appendix).

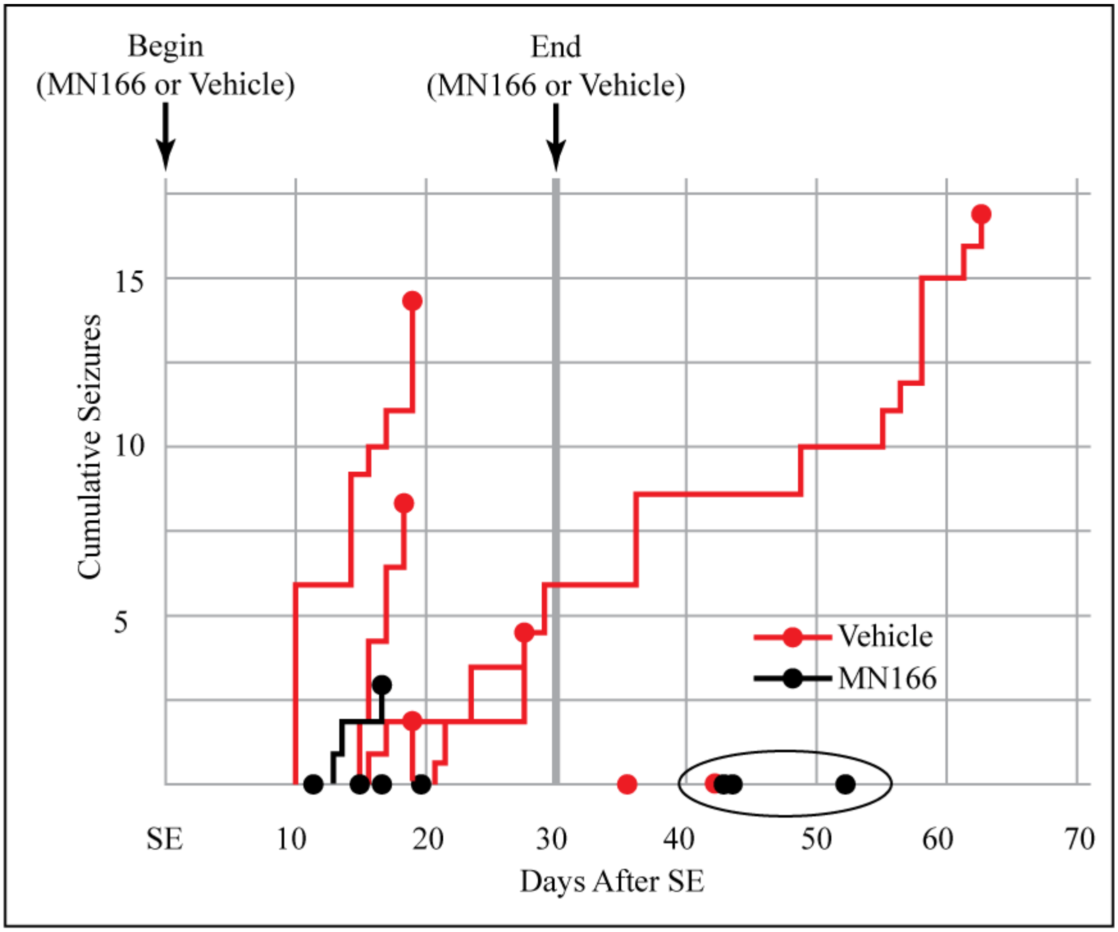


Fig. 5. Cumulatve seizures following SE in MN166 treated (black) and untreated Vehicle (red) rats.

Progress on objective 2:

Success with modifying the LFPI model so that it would yield short-term epileptiform spikes and seizures has taken longer than we initially planned. At the time of our first year progress report, we had discovered that Epileptiform Electrographic Events (EEE) previously associated exclusively with LFPI[1,2] were actually artifacts due to bruxing and eye boggling. We therefore changed our injury site to a more rostral location reported by Curia et al.[8] to yield higher success rates for spikes and eventual seizures. To our disappointment, the more rostral location performed no better. No animals displayed either epileptiform spikes or seizures, but all displayed theta activity (regarded as EEE by others), which we have now concluded is largely artifactual and seen as frequently in control as in injured animals. While this is a negative finding, it has important implications for the field, since there is now an ongoing controversy concerning what can be safely considered to be post-traumatic epileptiform activity in LFPI rats (see [2] and[5] for point/counter-point). All of our results challenge the findings of D'Ambrosio and colleagues and support the contention of Dudek and Bertram that most of what is being reported for LFPI is <u>not epilepsy</u>. Worse, because of this, treatments are being devised for preventing PTE (based on theta activity instead of spikes and seizures) that we feel are not necessarily related to seizures (epilepsy) as an endpoint (for a recent example of this, see [9]).

However, we have successfully changed our impact location based on work by Pitkanen's group[10] demonstrating that very severe impact LFPI injury <u>that includes entorhinal cortex</u> has a much higher chance of developing epileptiform spikes and seizures. Instead of using such severe impact, we moved our impact location caudally and ventrally to directly target entorhinal cortex (the cortical gateway to the hippocampus). This new location looks very promising in that we have begun to see epileptiform spikes (no seizures yet) from these animals, something we have not recorded in any of our medial or rostral impact locations. We also note that the hippocampal AEP at this new location is grossly altered from normal in the same way as our pilocarpine animal post-SE, with lower amplitude and prolonged temporal components. This discovery is very promising in that the entorhinal cortex has been demonstrated in humans and in other animal models of epilepsy to be directly involved in epileptogenesis. In humans, entorhinal cortex is on the inferior temporal lobe and highly exposed to potential trauma. In rats, this same area is in a caudal and ventral region which is difficult to access in vivo, perhaps explaining why it has been largely ignored in favor of more accessible dorsal locations by others studying LFPI.

Progress on objective 3:
We have therefore begun a study using the entorhinal impact location to examine the effects of MN166 on attenuating epileptogenesis, as monitored by AEP biomarkers, ESD and seizures. We will stick to MN166 treatment and not try SLC022 since we have had promising results with MN166 in the pilocarpine model and we need to get as large an N as possible with a single treatment regime. By the end of the final project year, we expect to have a full characterization of the entorhinal impact model of PTE and indications of the effect of anti-inflammatory treatment via MN166 on epileptogenesis.

Related additional studies:
1) As noted in our first project year progress report, we also made an unexpected serendipitous discovery concerning post-traumatic anxiety, that brain damaged animals displayed behaviors suggesting increased anxiety when placed in the recording chamber. We hypothesized that, regardless of the failure of this injury to reliably produce epilepsy, it did result in limbic hyperexcitability reflected in anxiety-like symptoms. In the first year, we found that glial attenuation with peri-injury administration of MN166 completely prevented development of post-traumatic anxiety. In this second year, we discovered that MN166 treatment, <u>delayed until one month post-injury when anxiety symptoms had completely developed</u>, had the effect of reversing post-traumatic anxiety. This was a surprising result suggesting that the neuroinflammatory sequelae to injury are enduring, representing a self-sustaining neuropathy that can be effectively extinguished with delayed glial modulation. This work has been submitted for publication (see Appendix). This work served as preliminary data for a proposal to the Department of Defense Broad Agency Announcement for Extramural Medical Research (see Appendix). The proposal was reviewed favorably and our responses to their memorandum for responses to external scientific review is presently under consideration.

2) We received a separate seed grant from the Autism Speaks Foundation to examine possible links between autism and epilepsy based on a common neuroinflammatory mechanism. Based on our experience in video/EEG epilepsy monitoring from the present project, we were able to perform far more advanced electrophysiology on this study than originally planned. The result in short was that we discovered a new (and the only) model of autism/epilepsy, a model that is based on a combination of the known maternal teratogens stress and terbutaline (typically taken to arrest preterm labor and closely linked to human autism). This result is quite exciting and is now the subject of an Idea Development proposal to the CDMRP Autism Research Program (see Appendix).

Recommended changes or future work to better address the research topic: Our goals for year three are in line with our SOW to explore the effectiveness of MN166 on PTE. We have added measurements of AEP biomarkers, which does not substantially change our objective. We are presently wrapping up the pilocarpine studies which were more productive and involved than originally expected. If these look like they will extend substantially into year three, we will submit a modified SOW, but at this point they do not.

KEY RESEARCH ACCOMPLISHMENTS:

- Completed and tested chronic video/EEG recording hardware and software.
- Developed high speed, random access, video/EEG review and analysis software.
- Achieved expertise in identifying normal and epileptiform EEG patterns in chronic recording.
- Discovered key discrepancy in current literature concerning “epileptiform” theta activity.
- Began pilocarpine model of temporal lobe epilepsy to test prevention of epileptogenesis.
- Discovered and published effect of glial attenuation on post-traumatic anxiety.
- Discovered a unique biomarker for epileptogenesis in the pilocarpine model using AEPs.
- Obtained promising preliminary results suggesting MN166 blocking of epileptogenesis in pilo model.
- Discovered that impact injuries to entorhinal cortex may be a very productive model of PTE.
- Discovered and are publishing effect of delayed glial attenuation on post-traumatic anxiety.
- Discovered a new (and the only) animal model of autism and epilepsy.

REPORTABLE OUTCOMES:

- Rodgers, K.M., Bercum, F.M., McCallum, D.L., Rudy, J.W., Frey, L.C., Johnson, K.W., Watkins, L.R. and Barth, D.S. Acute neuroimmune modulation attenuates the development of anxiety-like freezing behavior in an animal model of traumatic brain injury. *J. Neurotrauma, 2012, 29:1886-1897.*
- Barth, D.S. A Very High Speed System for Video/EEG Monitoring and Quantification of Post-traumatic Epileptogenesis. To be presented at the 2012 Military Health System Research Symposium to be held 13-16 August 2012 in Fort Lauderdale, Florida.
- Completed an Invention Disclosure Form so that our Technology Transfer Office can investigate whether the video/EEG review and analysis software is patentable or at least can be protected with a copyright.
- Alex Benison received his Ph.D. this year and Krista Rodgers will be receiving her Ph.D. this Fall. Both will be continuing on as a post-doctoral fellows on this project. The last year of their doctoral work was supported by this award.
- Submitted pre-proposal and then full proposal in response to a USAMRMC Broad Agency Announcement for a new project entitled: "The Prevention and Treatment of Post-traumatic Anxiety Through Neuroimmune Modulation", based on the serendipitous discovery made in the present project". Still under review.
- Rodgers, K.M., Bercum, F., Johnson, K., Watkins, L., Barth, D.S. 2012. Glial modulation with Ibudilast (MN166) attenuates neuroinflammation and autistic-like behaviors in the Terbutaline model of autism spectrum disorder (ASD) in rats. Program No. 443, New Orleans, LA: Society for Neuroscience.
- Submitted an Idea Development pre-proposal to the CDMRP Autism Research Program for a new project entitled: "ASD and Epilepsy: The First Animal Model to Examine Common Neuro-inflammatory Mechanisms and Neuro-immune Treatment". Under review.
- Submitted an Investigator-Initiated Research pre-proposal to the Peer Reviewed Medical Research Program for a new project entitled: "Sensory and Optogenetically Evoked Biomarkers to Study Post-traumatic Epileptogenesis". Under review.
- Submitted abstract for presentation at the annual meeting of the Society for Neuroscience entitled: "Hippocampal auditory evoked potentials in conjunction with continuous long-term video-EEG monitoring reveals novel biomarkers for epileptogenesis in the lithium-pilocarpine model of epilepsy in rats."
- Admitted 2 new graduate students, Florencia Bercum and Zachary Smith, who will be working on this project during the final year.

CONCLUSION:

Achievements: In the first year of this project, we constructed and tested all hardware for chronic video/EEG recording of 24 animals (expandable to 32). Software for logging video/EEG in a time-locked manner has been completed and is in use. Software for the extremely rapid quasi-random review and analysis of video/EEG data has been completed and is in use. We have been using this system to examine normal and brain damaged animals for the past several months. From this work we have discovered that what has been interpreted by others as pathological theta activity is also prominent in normal animals during "vacuous chewing" and we have entered into a very recent controversy in the field about what constitutes a valid post-traumatic epileptic seizure. We have developed a pilocarpine model of temporal lobe epilepsy and have begun recording epileptic spikes and seizures in this model. We will be conducting a study to determine if epileptogenesis (what goes on during the 1 month silent period before regular seizures evolve) can be attenuated or prevented with attenuation of glial activation using MN-166. Finally, we serendipitously discovered that LFPI produces post-traumatic anxiety that can be prevented with administration of MN-166 peri-injury.

In the second project year, we developed an auditory evoked potential biomarker for changes in brain excitability during the post-SE "silent period" and discovered progressive excitability changes continuing well past the first spontaneous seizure. We are now beginning the study to explore effects of glial attenuation with MN166 in blocking epileptogenesis in this model. Our further examination of more rostral impact locations in the LFPI model has led us to the firm conclusion that this model as it stands in not appropriate for studying PTE, at least temporal lobe epilepsy, which is always the locus of PTE in humans. However, we have had promising results using a far more caudal impact location over entorhinal cortex (the cortical gateway to the hippocampus). We will use this new model in the project year to examine effects of MN166 on short term spiking and PTE. We extended our serendipitous discovery of LFPI induced post-traumatic anxiety to demonstrating that we can reverse fully developed anxiety symptoms after they had fully developed using glial attenuation. Finally, we were able to leverage our experience with 24/7 epilepsy monitoring developed in this project to perform similar recording in a seed grant project from Autism Speaks to examine the relationship between autism and epilepsy in an animal model (the two pathologies are remarkably comorbid in the human population). This led to the recent discovery of a new (and only) animal model of autism/epilepsy based on combined maternal stress and terbutaline.

Recommendations: We recommend that the third year of this project include completion of the pilocarpine study, examining the effect of MN166 on attenuating epileptogenesis as measured by our new auditory evoked biomarkers as well as spiking and seizures. We also recommend proceeding with our examination of the effects of glial attenuation of PTE as planned but using our new (and far more realistic) entorhinal impact location.

So what: 1) Our data collection and analysis hardware/software comprises a unique and inexpensive approach to chronic monitoring of post-traumatic brain activity that is ideal, not just for the present research, but for emergency battlefield-related medical monitoring. For this reason, our results will be presented at this years MHSRS Symposium. Virtually nothing is known about epileptogenesis following traumatic brain injury in humans due in large part to the fact that video/EEG monitoring is rarely performed post-injury in the absence of a behavioral seizure. Our hardware/software system should be pursued as a tool for making this not only feasible but routine. 2) Our discovery that normal bruxing behavior in rats produces EEG activity closely resembling what has been identified as post-traumatic "pathological theta" turns out to be quite timely and important because there are currently attempts to use theta as a unique sign of early epileptogenesis and to develop drugs that might suppress this activity instead of waiting for development of actual seizures. This issue needs rapid resolution so the field of anti-epileptogenesis drugs does not head in the wrong direction. We are currently collaborating with epilepsy researcher, Dr. Edward Dudek at the University of Utah, on this effort and have developed a new entorhinal model that we hope will set the research back on course. 3) Our work with the pilocarpine model, while not directly related to post-traumatic epilepsy, could represent a major advance in the field if we are able to block or attenuate epileptogenesis in this more rapidly developing model. Our discovery of an auditory evoked biomarker to probe brain excitability changes during epileptogenesis will provide new insight into the effect of treatment in the pilocarpine and LFPI models, and should also be translatable to humans since auditory evoked potentials are easily recorded non-invasively. 5) Our unexpected finding that LFPI produces an animal model of post-traumatic anxiety, and that this development can be prevented by early attenuation of post-injury brain inflammation, may have extremely important implications for post-traumatic stress disorder (PTSD) experienced by many of our war fighters after head injury. It suggests that a strong component of PTSD may in fact be directly produced by the injury and not just the psychological setting within which it occurs. It also opens the way to potential future intervention. The additional discovery that PTSD-like behavior can be reversed by glial attenuation well after injury is perhaps even more important since it opens the possibility that developed PTSD in the veteran population may actually be treated. We are currently awaiting word from the DoD on funding to separately pursue this discovery.

REFERENCES:

1. D'Ambrosio R, Hakimian S, Stewart T, Verley DR, Fender JS, Eastman CL, et al. Functional definition of seizure provides new insight into post-traumatic epileptogenesis. Brain. 2009 Oct 1;132(Pt 10):2805–21.

2. D'Ambrosio R, Miller JW. What Is an Epileptic Seizure? Unifying Definitions in Clinical Practice and Animal Research to Develop Novel Treatments. Epilepsy Currents. 2010 May;10(3):61–6.

3. Rosales VP. Emotional stress and brux-like activity of the masseter muscle in rats. The European Journal of Orthodontics. 2002 Feb 1;24(1):107–17.

4. Zeredo J, Kumei Y, Shibazaki T, Yoshida N. Biting behavior induced by acute stress in the rat during experimental tooth movement. noldus.com

5. Dudek FE, Bertram EH. Counterpoint to "What Is an Epileptic Seizure?" By D'Ambrosio and Miller. Epilepsy Currents. 2010 Jul 8;10(4):91–4.

6. Curia G, Longo D, Biagini G, Jones RSG, Avoli M. The pilocarpine model of temporal lobe epilepsy. J. Neurosci. Methods. 2008 Jul 30;172(2):143–57. PMCID: PMC2518220

7. Rodgers KM, Bercum FM, McCallum DL, Rudy JW, Frey LC, Johnson KW, et al. Acute Neuroimmune Modulation Attenuates the Development of Anxiety-Like Freezing Behavior in an Animal Model of Traumatic Brain Injury. J. Neurotrauma. 2012 ed. 2012 Apr 26.

8. Curia G, Levitt M, Fender JS, Miller JW, Ojemann J, D apos Ambrosio R. Impact of Injury Location and Severity on Posttraumatic Epilepsy in the Rat: Role of Frontal Neocortex. Cereb Cortex. 2011 Jul 16;21(7):1574–92.

9. D'Ambrosio R, Eastman CL, Darvas F, Fender JS, Verley DR, Farin FM, et al. Mild passive focal cooling prevents epileptic seizures after head injury in rats. Ann Neurol. 2013 Feb;73(2):199–209. PMCID: PMC3608748

10. Kharatishvili I, Pitkanen A. Association of the severity of cortical damage with the occurrence of spontaneous seizures and hyperexcitability in an animal model of posttraumatic epilepsy. Epilepsy Res. 2010 Jun;90(1-2):47–59.

APPENDICES:

Attached are:

1) Copy of our recent manuscript concerning post-traumatic anxiety.

2) An abstract submitted to MHSRS reporting our video/EEG recording analysis system.

3) Abstract to be presented at this years Society for Neuroscience conference reporting results from our pilocarpine studies.

4) Idea Development proposal to the CDMRP Autism Research Program

5) Proposal to the Department of Defense Broad Agency Announcement for Extramural Medical Research to continue our work with preventing and treating PTSD.

6) Investigator-Initiated Research pre-proposal to Peer Reviewed Medical Research Program to develop our biomarkers for epileptogenesis using both auditory evoked responses and responses from optogenetic stimulation of hippocampus and auditory cortex.

7) Manuscript under review by J. Neurotramua concerning our recent discovery of delayed reversal of PTSD-like symptoms.

Journal of Neurotrauma

Journal of Neurotrauma: http://mc.manuscriptcentral.com/neurotrauma

Acute neuroimmune modulation attenuates the development of anxiety-like freezing behavior in an animal model of traumatic brain injury.

Journal:	*Journal of Neurotrauma*
Manuscript ID:	NEU-2011-2273.R1
Manuscript Type:	Regular Manuscript
Date Submitted by the Author:	24-Feb-2012
Complete List of Authors:	Rodgers, Krista; University of Colorado, Psychology and Neuroscience Bercum, Florencia; University of Colorado, Psychology and Neuroscience McCallum, Danielle; University of Colorado, Psychology and Neuroscience Rudy, Jerry; University of Colorado, Psychology and Neuroscience Frey, Lauren; University of Colorado Denver, Neurology Johnson, Kirk; MediciNova, Inc., Watkins, Linda; University of Colorado, Psychology and Neuroscience Barth, Daniel; University of Colorado, Psychology and Neuroscience
Keywords:	INFLAMMATION, TRAUMATIC BRAIN INJURY, ANIMAL STUDIES

Acute neuroimmune modulation attenuates the development of anxiety-like freezing behavior in an animal model of traumatic brain injury.

Krista M. Rodgers, M.A.[1], Florencia M. Bercum, B.A.[1], Danielle L. McCallum, B.A. [1], Jerry W. Rudy, Ph.D. [1], Lauren C. Frey, M.D.[2], Kirk W. Johnson, Ph.D.[3], Linda R. Watkins, Ph.D.[1] and Daniel S. Barth, Ph.D.[1]

[1]Department of Psychology and Neuroscience, University of Colorado, Boulder, CO, U.S.A., [2]Department of Neurology, University of Colorado Denver, and Colorado Injury Control Research Center, Denver, CO, U.S.A., [3]MediciNova, Inc., La Jolla, CA, U.S.A.

Running title: Neuroinflammation and post-traumatic anxiety.

Table of contents title: Neuroimmune modulation of anxiety behavior in a rat model of TBI.

Authors

Krista M. Rodgers

University of Colorado, Department of Psychology and Neuroscience, UCB 345

Boulder, CO 80309, USA

Phone: 303-492-0359, Fax: 303-492-2967, Email: Krista.Rodgers@colorado.edu

Florencia M. Bercum

University of Colorado, Department of Psychology and Neuroscience, UCB 345

Boulder, CO 80309, USA

Phone: 303-492-0359, Fax: 303-492-2967, Email: fbercum@gmail.com

Danielle L. McCallum

University of Colorado, Department of Psychology and Neuroscience, UCB 345

Boulder, CO 80309, USA

Phone: 303-492-0359, Fax: 303-492-2967, Email: Danielle.Mccallum@Colorado.EDU

Jerry W. Rudy

University of Colorado, Department of Psychology and Neuroscience, UCB 345

Boulder, CO 80309, USA

Phone: 303-492-3306, Fax: 303-492-2967, Email: Jrudy@colorado.edu

Lauren C. Frey

University of Colorado Denver School of Medicine, Department of Neurology

Aurora, CO 80045, USA

Phone: 720-848-2080, Email: Lauren.Frey@ucdenver.edu

Kirk W. Johnson

MediciNova, Inc.

4350 La Jolla Village Drive, Suite 950

La Jolla, CA, 92122, USA

Phone: 858-373-1500, Fax: 858-373-7000, Email: kjohnson@medicinova.com

Linda R. Watkins

University of Colorado, Department of Neurology

Boulder, CO 80309, USA

Phone: 303-492-7034, Fax: 303-492-2967, Email: Linda.Watkins@Colorado.EDU

Daniel S. Barth, PhD. (Corresponding author)

University of Colorado, Department of Psychology and Neuroscience, UCB 345

Boulder, CO 80309, USA

Phone: 303-492-0359, Fax: 303-492-2967, Email: dbarth@psych.colorado.edu

Abstract

Chronic anxiety is a common and debilitating result of traumatic brain injury in humans. While little is known about the neural mechanisms of this disorder, inflammation resulting from activation of the brain's immune response to insult has been implicated in both human post-traumatic anxiety and in recently developed animal models. In this study, we used a lateral fluid percussion injury (LFPI) model of traumatic brain injury in the rat and examined freezing behavior as a measure of post-traumatic anxiety. We found that LFPI produced anxiety-like freezing behavior accompanied by increased reactive gliosis (reflecting neuroimmune inflammatory responses) in key brain structures associated with anxiety: the amygdala, insula and hippocampus. Acute peri-injury administration of Ibudilast (MN166), a glial cell activation inhibitor, suppressed both reactive gliosis and freezing behavior, and continued neuroprotective effects were evidenced several months post-injury. These results support the conclusion that inflammation produced by neuroimmune responses to traumatic brain injury play a role in post-traumatic anxiety, and that acute suppression of injury-induced glial cell activation may have eventual promise for prevention of post-traumatic anxiety in humans.

Key Words

TBI, LFPI, PTSD, neuroinflammation

Introduction

The long-term consequences of traumatic brain injury (TBI) include heightened risk of neuropsychiatric disorders, of which anxiety disorders are the most prevalent (Rao and Lyketsos, 2000; Moore et al., 2006; Vaishnavi et al., 2009). Studies of the etiology of anxiety disorders implicate exaggerated responses of the amygdala and insula (Rauch et al., 1997; Simmons et al., 2006; Stein et al., 2007; Shin and Liberzon, 2010; Carlson et al., 2011), impaired inhibition of medial prefrontal cortex and anterior cingulate (Davidson, 2002; Shin et al., 2006; Milad et al., 2009; Shin and Liberzon, 2010) and decreased hippocampal volume (Bremner et al., 1995; Sapolsky, 2000; Shin et al., 2006). Yet, whether and how TBI induces neurochemical, structural, and functional abnormality in these structures is poorly understood.

There is increasing evidence that excessive inflammatory actions of the neuroimmune system may contribute to the development of anxiety disorders following TBI (Spivak et al., 1997; Gasque et al., 2000; Tucker et al., 2004; Shiozaki et al., 2005; von Känel et al., 2007; Hoge et al., 2009). Microglial cells are the first line of defense and primary immune effector cells in the CNS and respond immediately to even small pathological changes from damaged cells, producing proinflammatory cytokines and toxic molecules that compromise neuronal survival (Gehrmann, 1996; Gonzalez-Scarano and Baltuch, 1999; Aloisi, 2001; Town et al., 2005). This rapid microglial response often precedes the more delayed, yet prolonged activation of astrocytes and is thought to be involved with the onset and maintenance of astrogliosis (Graeber and Kreutzberg, 1988; McCann et al., 1996; Hanisch, 2002; Iravani et al., 2005; Herber et al., 2006; Zhang et al., 2010). It has been well established that microglia and astrocytes are activated during the innate immune response to brain injury, leading to the expression of high levels of proinflammatory cytokines, most notably interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α). While glial activation is typically neuroprotective (Aloisi, 2001; Farina et al., 2007), the chronic inflammatory responses and exaggerated proinflammatory cytokine levels observed following injury initiate neurotoxic processes resulting in secondary tissue damage (Gasque et al., 2000; Simi et al., 2007; Hailer, 2008; Lehnardt, 2010), neuronal death (Sternberg, 1997; Brown and Bal-Price, 2003; Schmidt et al., 2005; Beattie et al., 2010), secondary injury cascades (Bains and Shaw, 1997; Cernak et al., 2001b, a; Ansari et al., 2008a, b) and neuronal hyperexcitability (Hailer, 2008; Riazi et al., 2008; Rodgers et al., 2009; Beattie et al., 2010), all of which may contribute to the dysfunction of brain regions associated with anxiety.

Recently developed animal models of post-traumatic anxiety (O'Connor et al., 2003; Vink et al., 2003; Fromm et al., 2004; Sönmez et al., 2007; Wagner et al., 2007; Jones et al., 2008; Baratz et al., 2010; Liu et al., 2010) permit examination of the possible contributions of brain inflammation. Tests of post-traumatic anxiety in these models have typically included standard measurements of exploratory preference in mildly stressful environments, such as an open-field or elevated-plus testing apparatus. However, it has been frequently noted that measures of exploratory preference may be confounded by a marked overall decrease in exploration in brain-injured animals (O'Connor et al., 2003; Vink et al., 2003; Fromm et al., 2004). Decreased exploration cannot be attributed to TBI-induced motor deficits since numerous studies report only transient (~ 1 week) deficits following trauma (Yan et al., 1992; Taupin et al., 1993; Dixon et al., 1996; Fassbender et al., 2000; Goss et al., 2003; Cutler et al., 2005; Cutler et al., 2006b; Cutler et al., 2006a; Kline et al., 2007; Wagner et al., 2007; Bouilleret et al., 2009; Frey et al., 2009; Baratz et al., 2010; Liu et al., 2010). Rather, TBI-induced decreases in exploration have been attributed to the indirect effects of freezing (a primary component of the rodent's natural defensive behavior repertoire; Blanchard and Blanchard, 1988), suggesting an abnormally heightened response to stress in brain-injured rats (O'Connor et al., 2003; Vink et al., 2003; Fromm et al., 2004).

Based on these results, we tested the hypothesis that trauma-induced innate immune responses contribute to the development of anxiety-like behaviors in rats by directly examining freezing responses to a minor (novel environment) and major (foot-shock) stressor following Lateral Fluid Percussion Injury (LFPI; a clinically relevant animal model of human closed head injury). We also tested the effectiveness of a glial cell activation inhibitor, Ibudilast (MN166), in attenuating post-injury freezing behavior and reducing reactive gliosis in brain regions associated with hyperexcitability in anxiety disorders.

Materials and Methods

Sixty adult viral-free male Sprague-Dawley rats (275-325g; Harlan Laboratories, Madison, WI) were housed in pairs in temperature (23 ± 3 °C) and light (12:12 light:dark) controlled rooms with *ad libitum* access to food and water. All procedures were performed in accordance with University of Colorado Institutional Animal Care and Use Committee guidelines for the humane use of laboratory rats in biological research. Rats were randomly assigned to 1 of 10 groups (n = 6/group). Six groups (surgically naïve, sham operated, sham operated+vehicle, sham operated+MN166, LFPI+vehicle and LFPI+MN166) were shocked immediately after behavioral testing at 1 month post-surgery (sham operation or LFPI in the experimental rats). Surgically naïve rats received no injections or surgery, whereas sham operated rats received surgery but were not injected, the final 4 groups received sham or LFPI surgery and either vehicle injections or MN166 treatment. Another 4 groups (sham operated+vehicle, sham operated+MN166, LFPI+vehicle and LFPI+MN166) were run separately in a sucrose preference test to assess anhedonia (the inability to experience pleasure, a core symptom of human depression) without exposure to stressors (anxiety tests and foot shock).

Lateral Fluid Percussion Injury. LFPI rats were anesthetized with halothane (4% induction, 2.0-2.5% maintenance) and mounted in a stereotaxic frame. The lateral fluid percussion injury used in this study has been described previously (McIntosh et al., 1989; Thompson et al., 2005; Frey et al., 2009) utilizing a PV820 Pneumatic PicoPump (World Precision Instruments, Inc., Sarasota, FL) to deliver standardized pressure pulses of air to a standing column of fluid. A 3.0 mm diameter craniotomy was centered at 3 mm caudal to bregma and 4.0 mm lateral of the sagittal suture, with the exposed dura remaining intact. A female Luer-Loc hub (inside diameter of 3.5 mm) was secured over the craniotomy with cyanoacrylate adhesive. Following hub implantation, the animal was removed from the stereotaxic frame and connected to the LFPI apparatus. The LFPI apparatus delivered a moderate impact force (2.0 atmospheres; 10 ms). The injury cap was then removed, scalp sutured and the rats returned to their home cages for recovery. Sham operated rats underwent identical surgical preparation, but did not receive the brain injury.

Ibudilast (MN166) administration. MN166 (MediciNova, San Diego, CA) is a relatively non-selective phosphodiesterase inhibitor with anti-inflammatory actions via glial cell attenuation, which has been found to reduce glia-induced neuronal death through the suppression of nitric oxide, reactive oxygen species, and proinflammatory mediators (Mizuno et al., 2004; Rolan et al., 2009). Treated rats received a 5-day dosing regimen of once-daily MN166 injections (10 mg/kg, 1 ml/kg subcutaneously in corn oil) 24 hr prior to LFPI, the day of surgery and LFPI, and 3 days following LFPI. Weight was recorded prior to each dosing and treatment administered at the same time each day to maintain constant levels across a 24 hr period. Dose selection was based on prior animal pharmacology results (Ellis AL, SFN, 2008) showing MN166 to be safe and well tolerated, yielding plasma concentration-time profiles commensurate with high dose regimens in clinical development. MN166 administered via this regimen yields plasma and CNS concentrations that are linked to molecular target actions including, most potently, macrophage migration inhibitory factor (MIF) inhibition (Cho et al., 2010) and, secondarily, PDE's -4 and -10 inhibition (Gibson et al., 2006). The relevance of MIF inhibition in disorders of neuroimmune function such as neuropathic pain has recently been well demonstrated (Wang et al., 2011). Such dosing regimens have clearly been linked to glial attenuation in other animal models (Ledeboer et al., 2007), and the anti-inflammatory actions of

MN166 have recently been shown to suppress cerebral aneurysms in a dose-dependent manner (Yagi et al., 2010).

Tests of motor, vestibular and locomotive performance. Baseline testing of motor, vestibular and locomotive performance in all groups was conducted immediately prior to surgery and again, following a 1-week recovery period. These tests included ipsilateral and contralateral assessment of forelimb and hindlimb use to assess motor function, locomotion, limb use and limb preference (Bland et al., 2000; Bland et al., 2001), toe spread to assess gross motor response (Nitz et al., 1986), placing to assess visual and vestibular function (Schallert et al., 2000; Woodlee et al., 2005), catalepsy rod test to assess postural support and mobility (Sanberg et al., 1988), bracing to assess postural stability and catalepsy (Schallert et al., 1979; Morrissey et al., 1989) and air righting to assess dynamic vestibular function (Pellis et al., 1991b; Pellis et al., 1991a). Scoring ranged from 0 (severely impaired) to 5 (normal strength and function). The individual test scores were summed and a composite neuromotor score (0–45) was then generated for each animal. In addition to the composite neuromotor score, limb-use asymmetry was assessed during spontaneous exploration in the cylinder task, a common measure of motor forelimb function following central nervous system injury in rats (Schallert et al., 2000; Schallert, 2006) and post-injury locomotor activity was assessed through distance traveled on a running wheel, both tasks were scored for 5 minutes under red light (~90 lux).

Behavioral measures. A novel environment was used to assess freezing behavior in response to a minor stressor (Dellu et al., 1996). The environment consisted of a standard rat cage with one vertically and one horizontally striped wall. No aversive stimuli were introduced in this context and no conditioning occurred. Rats were tested (5 minutes) and the percent of freezing behavior was assessed. Freezing was defined as the absence of movement except for heart beat/respiration, and was recorded in 10 sec intervals.

Freezing behavior in the novel environment was measured before and after administration of a foot shock in a separate shock apparatus. The shock apparatus consisted of two chambers placed inside sound-attenuating chests. The floor of each chamber consisted of 18 stainless steel rods (4 mm diameter), spaced 1.5 cm center-to-center and wired to a shock generator and scrambler (Colbourn Instruments, Allentown, PA). An automated program delivered a 2-sec/1.5 mA electric shock. Rats were transported in black buckets and shocked immediately upon entry to chambers. Following shock, rats were returned to their home cages.

A sucrose preference test was also performed in separate groups of rats that did not receive foot-shock or testing in the novel environment. This task is commonly used to measure anhedonia in rodent models of depression (Monleon et al., 1995; Willner, 1997). The sucrose preference task was included because anxiety and depression share high rates of co-morbidity in humans (Moore et al., 2006) and was assessed as a possible confound to freezing behavior, due to possible co-occurrence of depression-like behavior. Rats were first habituated to sucrose solution, and were tested during the dark phase of the light/dark cycle to avoid the food and water deprivation necessary when testing during the light phase. Day 1 and day 2 consisted of habituation, day 3 and day 4 were baseline (averaged) and day 5 was the first test day. The rats were presented with two pre-weighted bottles containing 2% sucrose solution or tap water for a period of 4 hours. Thirty minutes into the task the bottles were swapped to force preference and counter for placement effects. Total sucrose intake and sucrose preference (sucrose intake/(sucrose intake + water intake * 100) were measured.

Timeline for behavioral testing: Following a 2-week recovery period from sham operation or LFPI in experimental animals, all groups except those to be evaluated for sucrose preference were tested in the novel context. Testing was performed at 2 weeks, 1, 2 and 3 months post-surgery. Shock was delivered after behavioral testing was completed at the 1 month time-point. Tests for sucrose preference were performed at 2 weeks, 1 month and 3 months post-surgery with no intervening foot-shock.

Immunohistochemistry: Immunoreactivity for OX-42 (targets CD11b/c, a marker of microglial activation) and glial fibrillary acidic protein (GFAP; a marker of astrocyte activation) was measured using an avidin-biotin-horseradish peroxidase (ABC) reaction (Loram et al., 2009). Brain sections (12 μm) were cut on a cryostat and mounted onto poly-L-lysine-coated slides and stored at -80 °C. Sections were post-fixed with 4% PFA for 15 min at room temperature, then treated with 0.03% H_2O_2 for 30 min at room temperature. The sections were incubated at 4 °C overnight in either mouse anti-rat OX-42 (1:100; BD Biosciences Pharmingen, San Jose, CA) or mouse anti-pig GFAP (1:100; MP Biomedicals, Aurora, OH). The next day, sections were incubated at room temperature for 2 h with biotinylated goat anti-mouse IgG antibody (1:200; Jackson ImmunoResearch, West Grove, PA). Sections were washed and incubated for 2 h at room temperature in ABC (1:400 Vector Laboratories, Burlingame, CA) and reacted with 3’, 3-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO). Glucose oxidase and β-D-glucose were used to generate hydrogen peroxide. Nickelous ammonium sulfate was added to the DAB solution to optimize the reaction product. Sections were air-dried over night and then dehydrated with graded alcohols, cleared in Histoclear and coverslipped with Permount (Fisher Scientific, Fairlawn, NJ). Densitometric analysis was performed using Scion Image software.

Image Analysis: Slides were viewed with an Olympus BX-61 microscope, using Olympus Microsuite software (Olympus America, Melville, NY) with bright-field illumination at 10X magnification. Images were opened in ImageJ, converted into gray scale and rescaled from inches to pixels. Background areas were chosen in the white matter or in cell-poor areas close to the region of interest (ROI). The number of pixels and the average pixel values above the set background were then computed and multiplied, giving an integrated densitometric measure (integrated gray level). Four measurements were made for each ROI; the measurements were then averaged to obtain a single integrated density value per rat, per region. All measurements were taken while blind to treatment group.

Statistical Analyses: Results are expressed as mean ± SEM. Analyses for all behavioral variables used analysis of variance (ANOVA) with repeated measures (time after injury), and treatment as the independent variable. The integrated density from the histology was only conducted at one time point and utilized one-way ANOVAs to compare regions between groups. Data were analyzed using SPSS® Statistics software and, in all cases, statistical significance was set at $p < 0.05$.

Results

Neuromotor composite scores of the brain-injured groups (LFPI+MN166, LFPI+vehicle) did not significantly differ from controls ($F(3,20) = 0.803$, $p = 0.508$). Rats in all groups consistently received normal scores on forelimb and hindlimb use, toe spread, placing, catalepsy rod, bracing, and air righting tests, indicating no impairments in motor, vestibular or locomotive functioning due to TBI. There were also no significant between group differences in limb-use asymmetry observed for contralateral ($F(5,29) = 0.544$, $p = 0.741$) and ipsilateral ($F(5,29) = 0.428$, $p = 0.826$) forelimb use during vertical exploratory behavior in the cylinder task, indicating no limb-use bias due to injury (Fig. 1A). No significant between group differences were found in locomotor performance evidenced by distance traveled during the running wheel activity ($F(5,29) = 0.069$, $p = 0.996$), revealing no post-injury impairments in locomotion (Fig. 1B). Nor were there significant between group differences in the sucrose preference task ($F(3,21) = 0.338$, $p = 0.798$), indicating no impairments in hedonic states post-injury.

Despite normal motor, vestibular and locomotive function, LFPI produced large increases in freezing behavior when rats were placed in a novel context (Fig. 2; $F(5,30) = 9.539$, $p < 0.0001$). Exposed only to this minor stressor (i.e. at 2 week and 1 month post-injury measurements conducted prior to shock), LPFI rats injected with either MN166 or vehicle (Fig. 2; white and black bars, respectively) froze approximately twice as long as naïve or sham operated rats (Fig. 2; light and dark grey bars, respectively; $p < 0.01$). At 2 and 3-month measurement times, following the additional major stressor of shock (Fig. 2; arrows), freezing in both naïve and sham operated rats remained constant at approximately 10%. Freezing in LFPI rats treated with MN166 remained consistently higher than these controls ($p < 0.001$), but, while appearing higher compared to earlier post-injury measurements in the same animals, this increased freezing compared to naïve and sham operated rats before (1 month) and following (2 month) shock did not reach significance ($p=0.316$). By contrast, LFPI+vehicle rats nearly doubled their freezing time to approximately 50% (Fig. 2; black bars) compared to pre-shock values ($p < 0.001$), freezing approximately twice as long as LFPI+MN166 rats ($p < 0.001$) and 5 times as long as naïve and sham operated controls ($p < 0.001$) at the 2 and 3 month post-injury measurement times.

The behavioral effects of injections alone, independent of LFPI, are reflected in sham surgery groups with injections of either MN166 or vehicle (Fig. 2; narrow and broad diagonal lines, respectively). Sham operated rats tended to freeze more than un-injected naïve and sham operated controls, reaching significance for both groups at the 2 and 3-month measurement points ($p < 0.01$) and suggesting that injections alone are aversive and can contribute to subsequent freezing. However, even at pre-shock measurement points, LFPI animals that received the same injections of MN166 or vehicle froze significantly more than injected controls ($p < 0.01$), indicating substantial enhancement of freezing produced by LFPI. This effect became more apparent following shock, where LFPI+vehicle rats froze twice as long as the injected controls ($p < 0.001$). By contrast, LFPI+MN166 rats were not distinguishable from either injected control group following shock, suggesting that their elevated freezing compared to naïve and sham operated animals was the result of injections alone and that MN166 eliminated the exaggerated freezing response to shock characterizing LFPI+vehicle rats.

OX-42 and GFAP immunoreactivity (reflecting microglia and astrocytic activation) was assessed in the insula, amygdala and hippocampus in brain-injured rats for comparison to sham operated and surgically naïve rats. Representative images (40X), showing GFAP immunoreactivity in several of these regions, are shown in Figure 3, revealing normal astrocyte morphology in surgically naïve and sham operated rats. LFPI+vehicle rats showed clear signs of reactive astrocytes (Fig. 3; bottom row). LFPI rats treated with MN166 (Fig. 3; third row) were difficult to differentiate from sham operated or surgically naïve control groups.

Densitometry of GFAP labeling in all areas examined confirmed that activation of astrocytes was significantly greater in LFPI compared to all other groups in insula (Fig. 4A; left bars; $F(3,19) = 13.17$, $p < 0.0001$), amygdala (Fig. 4B; left bars; $F(3,18) = 7.54$, $p < 0.002$) and hippocampus (Fig. 4C; left bars; $F(3,15) = 8.47$, $p < 0.002$). In contrast, no differences in GFAP labeling were observed between surgically naïve, sham operated and LFPI+MN166 groups in any of the regions examined. While MN166 treated LFPI rats were not distinguishable from surgically naïve or sham operated controls, post-hoc analyses revealed that LFPI+vehicle rats had significantly greater astrocyte activation in all 3 brain regions as compared to controls (Fig. 4A-C): insula ($p < 0.002$ vs. surgically naive, sham operated and LFPI+MN166), amygdala ($p < 0.02$ vs. surgically naive, sham operated and LFPI+MN166) and hippocampus ($p < 0.03$ vs. surgically naive, sham operated and LFPI+MN166).

Analysis of GFAP immunoreactivity in sub-regions of the insula (Fig. 4A; right bars), amygdala (Fig. 4B; right bars), and hippocampus (Fig. 4C; right bars), also revealed no differences between surgically naïve, sham operated and LFPI+MN166 groups. As in the regional analysis, LFPI+vehicle rats showed increased astrocyte activation over controls in most sub-regions examined. In the insula, LFPI+vehicle rats showed significantly increased GFAP labeling in agranular ($F(3,19) = 16.778$, $p < 0.0001$), dysgranular ($F(3,19) = 6.042$, $p < 0.005$) and granular ($F(3,19) = 5.277$, $p < 0.008$) regions, as compared to control groups. In the amygdala, GFAP labeling in LFPI+vehicle rats was significantly increased in the BLA ($F(3,18) = 4.050$, $p < 0.023$) and CE ($F(3,18) = 5.012$, $p < 0.011$) nuclei, as compared to controls. LFPI+vehicle rats also showed increased GFAP expression in the hippocampus, but this was only significant in CA3 ($F(3,18) = 3.810$, $p < 0.03$) and approached significance in CA1 ($F(3,17) = 3.234$, $p = 0.055$).

LFPI+vehicle rats also showed significantly increased microglia activation compared to control groups, as measured by OX-42 labeling, but this was restricted to the insula (Fig. 4D; $F(3,19) = 5.59$, $p < 0.007$). Analysis of sub-regions of the insula also revealed increases in microglial activation for LFPI+vehicle rats, and post-hoc comparisons showed that LFPI alone significantly increased OX-42 labeling in agranular ($F(3,19) = 11.186$, $p < 0.0001$), granular

($F(3,18) = 3.740$, $p < 0.03$), and approaching significance ($F(3,19) = 2.742$, $p < 0.072$) in dysgranular areas. No differences in OX-42 labeling were observed between surgically naïve, sham operated and LFPI+MN166 groups in any insular regions examined. No significant between group differences were found in OX-42 expression for the amygdala or hippocampus.

Discussion

These data suggest a link between injury-induced brain inflammation and post-traumatic anxiety. Rats with LFPI display freezing responses to the minor stress of a novel environment that is 2-3 times normal and which, unlike controls, is nearly doubled by the delivery of a major foot-shock stressor. LFPI also results in marked reactive gliosis in brain regions associated with anxiety. The possibility that post-traumatic brain inflammation and gliosis may contribute to anxiety-like behavior observed here, is supported by the effects of glial-cell activation inhibitor MN166. MN166 reduces reactive gliosis and TBI-induced freezing behavior, rendering these animals histologically and behaviorally indistinguishable from naïve and sham operated controls. To our knowledge, this is the first study to report pharmacological immunosuppression resulting in the reduction of anxiety-like behaviors following TBI.

A possible mechanism for neuroimmune induced post-traumatic anxiety.

Our finding of prolonged reactive gliosis in brain structures including, but likely not confined to, the hippocampus, amygdala and insular cortex, suggests that these structures may contribute to the persistent enhanced freezing of our brain-injured animals in reaction to a novel environment. All three structures have been implicated in rodent research investigating the pathogenesis of anxiety (Davis, 1992; Davis et al., 1994; Davidson, 2002; Vyas et al., 2004; Paulus and Stein, 2006; Rauch et al., 2006; Canteras et al., 2010) and fear behavior in the rat (Sullivan, 2004; Rosen and Donley, 2006; Milad et al., 2009; Liu et al., 2010).

The mechanisms by which immune responses may contribute to dysfunction of these structures remain to be determined. It is well established that LFPI in the rat results in activation of microglia and astrocytes as part of the innate immune response to insult. A number of studies indicate that LFPI-induced reactive gliosis follows a distinct time-course, beginning with predominant microglia activation that peaks within a week (Hill et al., 1996; Nonaka et al., 1999; Grady et al., 2003; Gueorguieva et al., 2008; Clausen et al., 2009; Yu et al., 2010) but continues for several weeks and overlaps later with persistent astrocytic activation (D'Ambrosio et al., 2004; Yu et al., 2010). Microglia are resident macrophages and first responders to pathogens and neuronal insults in the CNS. They react rapidly, leading to activation of astrocytes and prolonged disruption of neuronal function (Iravani et al., 2005; Herber et al., 2006; Zhang et al., 2009; Zhang et al., 2010). Several lesion paradigms have also shown rapid microglial response followed by delayed astrocyte reaction (Gehrmann et al., 1991; Dusart and Schwab, 1994; Frank and Wolburg, 1996; McCann et al., 1996; Liberatore et al., 1999).

Our results support this well-documented temporal relationship suggesting that microglial activation precedes astrocytic activation and plays a role in the onset and maintenance of astrogliosis (Graeber and Kreutzberg, 1988; McCann et al., 1996; Hanisch, 2002; Iravani et al., 2005; Herber et al., 2006; Zhang et al., 2010). This time-course is consistent with behavioral freezing responses in the present study, appearing rapidly within 2 weeks but persisting unabated for the 3-month post-injury measurement period. It is also consistent with our immunohistochemistry results, indicating injury-induced astrocytic activation in all 3 regions of interest, insula, amygdala and hippocampus at 3 months post-injury, but less activation of microglia, only significant in the insula. The lower levels of microglia expression are likely due to assessment at 3 months post-injury.

Trauma-related reactive gliosis is well known to result in the release of high levels of pro-inflammatory cytokines, specifically tumor necrosis factor-α (TNF-α) (Taupin et al., 1993; Fan et al., 1996; Lloyd et al., 2008), interleukin-1 beta (IL-1β) (Taupin et al., 1993; Fan et al., 1995; Fassbender et al., 2000; Yan et al., 2002; Lloyd et al., 2008), and interleukin-6 (IL-6; (Taupin et al., 1993; Yan et al., 2002; Lloyd et al., 2008), which are central mediators of neuroinflammation following head injury (Fan et al., 1995; Rothwell and Hopkins, 1995; Rothwell and Strijbos, 1995; Fan et al., 1996; Simi et al., 2007). Release of these pro-inflammatory cytokines, particularly IL-1β and TNF-α, pathologically increases neuronal excitability in all brain regions where it has been measured (Riazi et al., 2008; Schafers and Sorkin, 2008; Rodgers et al., 2009; Beattie et al., 2010; Maroso et al., 2010). While neuronal excitability and proinflammatory cytokine levels were not measured in the present study, neuroinflammation has been implicated in neuronal excitability of amygdala and insular cortex and anxiety-like behavior by others using c-Fos labeling (Abrous et al., 1999; Ikeda et al., 2003; Kung et al., 2010). These same regions have also consistently been reported to be hyperexcitable in human imaging data across a variety of anxiety disorders (Rauch et al., 1997; Shin et al., 2006; Simmons et al., 2006; Stein et al., 2007; Shin and Liberzon, 2010; Carlson et al., 2011).

Attenuation of post-traumatic anxiety with MN166.

Meta-analysis of the impact of pharmacological treatments on behavioral, cognitive, and motor outcomes after traumatic brain injury in rodent models (Wheaton et al., 2011) indicates that of 16 treatment strategies evaluated to date, improved cognition and motor function have been reported, but almost no treatments have improved behaviors related to psychiatric dysfunction in general and anxiety in specific. Exceptions to this are recent promising reports of treatments such as magnesium sulphate to limit excitotoxic damage (Vink et al., 2003; Fromm et al., 2004; O'Connor, 2003, 533-41) and resevatrol to limit excitotoxicity, ischemia, hypoxia (Sönmez et al., 2007), both increasing open field exploration (resulting from decreased freezing) and therefore presumably decreasing post-injury anxiety.

Glial targeted immunosuppression has also been found to be neuroprotective following TBI in rodents, resulting in increased structural preservation and improved functional outcomes (Hailer, 2008); including recent reports that MN166 significantly attenuated brain edema formation, cerebral atrophy and apoptosis in neuronal cells following ischemic brain injury in rats, increasing neuronal survival rates (Lee et al., 2011). MN166 may reduce neuronal damage in regions involved in anxiety, mitigating the role of glial activation, neurotoxicity and hyperexcitability in the subsequent development of anxiety-like behaviors. While not focused on post-traumatic anxiety, MN166 has been found to reduce intracellular calcium accumulation (Yanase et al., 1996), apoptosis, functional damage and passive avoidance behaviors following a transient ischemia model in rats (Yoshioka et al., 2002). Increasing evidence supports neuroinflammation, chronic inflammatory responses, proinflammatory cytokines, neuronal hyperexcitability, and secondary injury cascades in the pathophysiology of post-traumatic anxiety. The mechanisms of the effect of MN166 on TBI-induced anxiety-like behavior are not fully known. However, the results of this study provide evidence of a neuroprotective role for MN166 in attenuating and perhaps preventing development of post-traumatic anxiety.

Further establishing a relationship between TBI, neuroimmune responses, neurocircuitry and anxiety disorders, is important to further understand the sequelae of TBI and to the development of effective treatment strategies. The development of anxiety disorders following TBI is a complex and multifaceted problem, and finding treatments that work will require multifaceted approaches. The injury itself initiates many complex biological events including glial activation, breakdown of the blood brain barrier, excitotoxicity and chronic neuroinflammation. While primary injury often cannot be prevented, it may be possible to reduce secondary injury, leading to better functional and behavioral recovery following TBI. The present results, using peri-injury treatment with MN166 to prevent post-traumatic freezing behavior, not only suggest a role for neuroimmune inflammation in anxiety physiology, but similarly successful results with post-injury treatment could introduce a promising and clinically realistic translational possibility for prevention of post-traumatic anxiety in humans.

Acknowledgements

US Army Medical Research and Material Command grant PR100040, Craig Hospital Gift Fund, University of Colorado Innovative Seed Grant, Autism Speaks Pilot Study grant 7153, and National Institutes of Health grant NS36981 to DSB, and National Institutes of Health grants DA024044, DA01767 to LRW.

Author Disclosure Statement

Krista M. Rodgers: No competing financial interests exist.

Florencia M. Bercum: No competing financial interests exist.

Danielle L. McCallum: No competing financial interests exist.

Jerry W. Rudy: No competing financial interests exist.

Lauren C. Frey: No competing financial interests exist.

Kirk W. Johnson: Chief science officer of MediciNova, Inc., the pharmaceutical firm providing MN166 for this research.

Linda R. Watkins: No competing financial interests exist.

Daniel S. Barth: No competing financial interests exist.

References

Abrous DN, Rodriguez J, le Moal M, Moser PC, Barneoud P (1999) Effects of mild traumatic brain injury on immunoreactivity for the inducible transcription factors c-Fos, c-Jun, JunB, and Krox-24 in cerebral regions associated with conditioned fear responding. Brain research 826:181-192.

Aloisi F (2001) Immune function of microglia. Glia 36:165-179.

Ansari MA, Roberts KN, Scheff SW (2008a) Oxidative stress and modification of synaptic proteins in hippocampus after traumatic brain injury. Free Radic Biol Med 45:443-452.

Ansari MA, Roberts KN, Scheff SW (2008b) A time course of contusion-induced oxidative stress and synaptic proteins in cortex in a rat model of TBI. J Neurotrauma 25:513-526.

Bains JS, Shaw CA (1997) Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. Brain Res Brain Res Rev 25:335-358.

Baratz R, Rubovitch V, Frenk H, Pick CG (2010) The influence of alcohol on behavioral recovery after mTBI in mice. J Neurotrauma 27:555-563.

Beattie MS, Ferguson AR, Bresnahan JC (2010) AMPA-receptor trafficking and injury-induced cell death. Eur J Neurosci 32:290-297.

Bland ST, Pillai RN, Aronowski J, Grotta JC, Schallert T (2001) Early overuse and disuse of the affected forelimb after moderately severe intraluminal suture occlusion of the middle cerebral artery in rats. Behav Brain Res 126:33-41.

Bland ST, Schallert T, Strong R, Aronowski J, Grotta JC (2000) Early exclusive use of the affected forelimb after moderate transient focal ischemia in rats : functional and anatomic outcome. Stroke 31:1144-1152.

Bouilleret V, Cardamone L, Liu YR, Fang K, Myers DE, O'Brien TJ (2009) Progressive brain changes on serial manganese-enhanced MRI following traumatic brain injury in the rat. J Neurotrauma 26:1999-2013.

Bremner JD, Randall P, Scott TM, Bronen RA, Seibyl JP, Southwick SM, Delaney RC, McCarthy G, Charney DS, Innis RB (1995) MRI-based measurement of hippocampal volume in patients with combat-related posttraumatic stress disorder. Am J Psychiatry 152:973-981.

Brown GC, Bal-Price A (2003) Inflammatory neurodegeneration mediated by nitric oxide, glutamate, and mitochondria. Mol Neurobiol 27:325-355.

Canteras NS, Resstel LB, Bertoglio LJ, Carobrez Ade P, Guimaraes FS (2010) Neuroanatomy of anxiety. Curr Top Behav Neurosci 2:77-96.

Carlson JM, Greenberg T, Rubin D, Mujica-Parodi LR (2011) Feeling anxious: anticipatory amygdalo-insular response predicts the feeling of anxious anticipation. Soc Cogn Affect Neurosci 6:74-81.

Cernak I, Wang Z, Jiang J, Bian X, Savic J (2001a) Ultrastructural and functional characteristics of blast injury-induced neurotrauma. J Trauma 50:695-706.

Cernak I, Wang Z, Jiang J, Bian X, Savic J (2001b) Cognitive deficits following blast injury-induced neurotrauma: possible involvement of nitric oxide. Brain Inj 15:593-612.

Cho Y, Crichlow GV, Vermeire JJ, Leng L, Du X, Hodsdon ME, Bucala R, Cappello M, Gross M, Gaeta F, Johnson K, Lolis EJ (2010) Allosteric inhibition of macrophage migration inhibitory factor revealed by ibudilast. Proc Natl Acad Sci U S A 107:11313-11318.

Clausen F, Hanell A, Bjork M, Hillered L, Mir AK, Gram H, Marklund N (2009) Neutralization of interleukin-1beta modifies the inflammatory response and improves histological and cognitive outcome following traumatic brain injury in mice. Eur J Neurosci 30:385-396.

Cutler SM, Vanlandingham JW, Stein DG (2006a) Tapered progesterone withdrawal promotes long-term recovery following brain trauma. Exp Neurol 200:378-385.

Cutler SM, Pettus EH, Hoffman SW, Stein DG (2005) Tapered progesterone withdrawal enhances behavioral and molecular recovery after traumatic brain injury. Exp Neurol 195:423-429.

Cutler SM, VanLandingham JW, Murphy AZ, Stein DG (2006b) Slow-release and injected progesterone treatments enhance acute recovery after traumatic brain injury. Pharmacol Biochem Behav 84:420-428.

D'Ambrosio R, Fairbanks JP, Fender JS, Born DE, Doyle DL, Miller JW (2004) Post-traumatic epilepsy following fluid percussion injury in the rat. Brain 127:304-314.

Davidson RJ (2002) Anxiety and affective style: role of prefrontal cortex and amygdala. Biological psychiatry 51:68-80.

Davis M (1992) The role of the amygdala in fear and anxiety. Annu Rev Neurosci 15:353-375.

Davis M, Rainnie D, Cassell M (1994) Neurotransmission in the rat amygdala related to fear and anxiety. Trends Neurosci 17:208-214.

Dellu F, Mayo W, Vallee M, Maccari S, Piazza PV, Le Moal M, Simon H (1996) Behavioral reactivity to novelty during youth as a predictive factor of stress-induced corticosterone secretion in the elderly--a life-span study in rats. Psychoneuroendocrinology 21:441-453.

Dixon CE, Bao J, Long DA, Hayes RL (1996) Reduced evoked release of acetylcholine in the rodent hippocampus following traumatic brain injury. Pharmacol Biochem Behav 53:679-686.

Dusart I, Schwab ME (1994) Secondary cell death and the inflammatory reaction after dorsal hemisection of the rat spinal cord. Eur J Neurosci 6:712-724.

Ellis AL WJ, Brown K, Blackwood C, Ramos K, Starnes C, Maier SF, and Watkins LR (SFN, 2008) Characterization of exaggerated pain behavior and glial activation in a novel rat model of spinal cord injury.

Fan L, Young PR, Barone FC, Feuerstein GZ, Smith DH, McIntosh TK (1995) Experimental brain injury induces expression of interleukin-1 beta mRNA in the rat brain. Brain Res Mol Brain Res 30:125-130.

Fan L, Young PR, Barone FC, Feuerstein GZ, Smith DH, McIntosh TK (1996) Experimental brain injury induces differential expression of tumor necrosis factor-alpha mRNA in the CNS. Brain Res Mol Brain Res 36:287-291.

Farina C, Aloisi F, Meinl E (2007) Astrocytes are active players in cerebral innate immunity. Trends Immunol 28:138-145.

Fassbender K, Schneider S, Bertsch T, Schlueter D, Fatar M, Ragoschke A, Kuhl S, Kischka U, Hennerici M (2000) Temporal profile of release of interleukin-1beta in neurotrauma. Neurosci Lett 284:135-138.

Frank M, Wolburg H (1996) Cellular reactions at the lesion site after crushing of the rat optic nerve. Glia 16:227-240.

Frey LC, Hellier J, Unkart C, Lepkin A, Howard A, Hasebroock K, Serkova N, Liang L, Patel M, Soltesz I, Staley K (2009) A novel apparatus for lateral fluid percussion injury in the rat. J Neurosci Methods 177:267-272.

Fromm L, Heath DL, Vink R, Nimmo AJ (2004) Magnesium attenuates post-traumatic depression/anxiety following diffuse traumatic brain injury in rats. J Am Coll Nutr 23:529S-533S.

Gasque P, Dean YD, McGreal EP, VanBeek J, Morgan BP (2000) Complement components of the innate immune system in health and disease in the CNS. Immunopharmacology 49:171-186.

Gehrmann J (1996) Microglia: a sensor to threats in the nervous system? Res Virol 147:79-88.

Gehrmann J, Schoen SW, Kreutzberg GW (1991) Lesion of the rat entorhinal cortex leads to a rapid microglial reaction in the dentate gyrus. A light and electron microscopical study. Acta Neuropathol 82:442-455.

Gibson LC, Hastings SF, McPhee I, Clayton RA, Darroch CE, Mackenzie A, Mackenzie FL, Nagasawa M, Stevens PA, Mackenzie SJ (2006) The inhibitory profile of Ibudilast against the human phosphodiesterase enzyme family. Eur J Pharmacol 538:39-42.

Gonzalez-Scarano F, Baltuch G (1999) Microglia as mediators of inflammatory and degenerative diseases. Annu Rev Neurosci 22:219-240.

Goss CW, Hoffman SW, Stein DG (2003) Behavioral effects and anatomic correlates after brain injury: a progesterone dose-response study. Pharmacol Biochem Behav 76:231-242.

Grady MS, Charleston JS, Maris D, Witgen BM, Lifshitz J (2003) Neuronal and glial cell number in the hippocampus after experimental traumatic brain injury: analysis by stereological estimation. J Neurotrauma 20:929-941.

Graeber MB, Kreutzberg GW (1988) Delayed astrocyte reaction following facial nerve axotomy. J Neurocytol 17:209-220.

Gueorguieva I, Clark SR, McMahon CJ, Scarth S, Rothwell NJ, Tyrrell PJ, Hopkins SJ, Rowland M (2008) Pharmacokinetic modelling of interleukin-1 receptor antagonist in plasma and cerebrospinal fluid of patients following subarachnoid haemorrhage. Br J Clin Pharmacol 65:317-325.

Hailer NP (2008) Immunosuppression after traumatic or ischemic CNS damage: it is neuroprotective and illuminates the role of microglial cells. Prog Neurobiol 84:211-233.

Hanisch UK (2002) Microglia as a source and target of cytokines. Glia 40:140-155.

Herber DL, Maloney JL, Roth LM, Freeman MJ, Morgan D, Gordon MN (2006) Diverse microglial responses after intrahippocampal administration of lipopolysaccharide. Glia 53:382-391.

Hill SJ, Barbarese E, McIntosh TK (1996) Regional heterogeneity in the response of astrocytes following traumatic brain injury in the adult rat. J Neuropathol Exp Neurol 55:1221-1229.

Hoge EA, Brandstetter K, Moshier S, Pollack MH, Wong KK, Simon NM (2009) Broad spectrum of cytokine abnormalities in panic disorder and posttraumatic stress disorder. Depress Anxiety 26:447-455.

Ikeda K, Onaka T, Yamakado M, Nakai J, Ishikawa TO, Taketo MM, Kawakami K (2003) Degeneration of the amygdala/piriform cortex and enhanced fear/anxiety behaviors in sodium pump alpha2 subunit (Atp1a2)-deficient mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:4667-4676.

Iravani MM, Leung CC, Sadeghian M, Haddon CO, Rose S, Jenner P (2005) The acute and the long-term effects of nigral lipopolysaccharide administration on dopaminergic dysfunction and glial cell activation. Eur J Neurosci 22:317-330.

Jones NC, Cardamone L, Williams JP, Salzberg MR, Myers D, O'Brien TJ (2008) Experimental traumatic brain injury induces a pervasive hyperanxious phenotype in rats. Journal of neurotrauma 25:1367-1374.

Kline AE, Wagner AK, Westergom BP, Malena RR, Zafonte RD, Olsen AS, Sozda CN, Luthra P, Panda M, Cheng JP, Aslam HA (2007) Acute treatment with the 5-HT(1A) receptor agonist 8-OH-DPAT and chronic environmental enrichment confer neurobehavioral benefit after experimental brain trauma. Behav Brain Res 177:186-194.

Kung JC, Chen TC, Shyu BC, Hsiao S, Huang AC (2010) Anxiety- and depressive-like responses and c-fos activity in preproenkephalin knockout mice: oversensitivity hypothesis of enkephalin deficit-induced posttraumatic stress disorder. J Biomed Sci 17:29.

Ledeboer A, Hutchinson MR, Watkins LR, Johnson KW (2007) Ibudilast (AV-411). A new class therapeutic candidate for neuropathic pain and opioid withdrawal syndromes. Expert Opin Investig Drugs 16:935-950.

Lee JY, Cho E, Ko YE, Kim I, Lee KJ, Kwon SU, Kang DW, Kim JS (2011) Ibudilast, a phosphodiesterase inhibitor with anti-inflammatory activity, protects against ischemic brain injury in rats. Brain research.

Lehnardt S (2010) Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. Glia 58:253-263.

Liberatore GT, Jackson-Lewis V, Vukosavic S, Mandir AS, Vila M, McAuliffe WG, Dawson VL, Dawson TM, Przedborski S (1999) Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. Nat Med 5:1403-1409.

Liu YR, Cardamone L, Hogan RE, Gregoire MC, Williams JP, Hicks RJ, Binns D, Koe A, Jones NC, Myers DE, O'Brien TJ, Bouilleret V (2010) Progressive metabolic and structural cerebral perturbations after traumatic brain injury: an in vivo imaging study in the rat. J Nucl Med 51:1788-1795.

Lloyd E, Somera-Molina K, Van Eldik LJ, Watterson DM, Wainwright MS (2008) Suppression of acute proinflammatory cytokine and chemokine upregulation by post-injury administration of a novel small molecule improves long-term neurologic outcome in a mouse model of traumatic brain injury. J Neuroinflammation 5:28.

Loram LC, Harrison JA, Sloane EM, Hutchinson MR, Sholar P, Taylor FR, Berkelhammer D, Coats BD, Poole S, Milligan ED, Maier SF, Rieger J, Watkins LR (2009) Enduring reversal of neuropathic pain by a single intrathecal injection of adenosine 2A receptor agonists: a novel therapy for neuropathic pain. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:14015-14025.

Maroso M, Balosso S, Ravizza T, Liu J, Aronica E, Iyer AM, Rossetti C, Molteni M, Casalgrandi M, Manfredi AA, Bianchi ME, Vezzani A (2010) Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nat Med 16:413-419.

McCann MJ, O'Callaghan JP, Martin PM, Bertram T, Streit WJ (1996) Differential activation of microglia and astrocytes following trimethyl tin-induced neurodegeneration. Neuroscience 72:273-281.

McIntosh TK, Vink R, Noble L, Yamakami I, Fernyak S, Soares H, Faden AL (1989) Traumatic brain injury in the rat: characterization of a lateral fluid-percussion model. Neuroscience 28:233-244.

Milad MR, Pitman RK, Ellis CB, Gold AL, Shin LM, Lasko NB, Zeidan MA, Handwerger K, Orr SP, Rauch SL (2009) Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. Biological psychiatry 66:1075-1082.

Mizuno T, Kurotani T, Komatsu Y, Kawanokuchi J, Kato H, Mitsuma N, Suzumura A (2004) Neuroprotective role of phosphodiesterase inhibitor ibudilast on neuronal cell death induced by activated microglia. Neuropharmacology 46:404-411.

Monleon S, D'Aquila P, Parra A, Simon VM, Brain PF, Willner P (1995) Attenuation of sucrose consumption in mice by chronic mild stress and its restoration by imipramine. Psychopharmacology (Berl) 117:453-457.

Moore EL, Terryberry-Spohr L, Hope DA (2006) Mild traumatic brain injury and anxiety sequelae: a review of the literature. Brain injury : [BI] 20:117-132.

Morrissey TK, Pellis SM, Pellis VC, Teitelbaum P (1989) Seemingly paradoxical jumping in cataleptic haloperidol-treated rats is triggered by postural instability. Behav Brain Res 35:195-207.

Nitz AJ, Dobner JJ, Matulionis DH (1986) Pneumatic tourniquet application and nerve integrity: motor function and electrophysiology. Exp Neurol 94:264-279.

Nonaka M, Chen XH, Pierce JE, Leoni MJ, McIntosh TK, Wolf JA, Smith DH (1999) Prolonged activation of NF-kappaB following traumatic brain injury in rats. J Neurotrauma 16:1023-1034.

O'Connor CA, Cernak I, Vink R (2003) Interaction between anesthesia, gender, and functional outcome task following diffuse traumatic brain injury in rats. J Neurotrauma 20:533-541.

Paulus MP, Stein MB (2006) An insular view of anxiety. Biological psychiatry 60:383-387.

Pellis SM, Whishaw IQ, Pellis VC (1991a) Visual modulation of vestibularly-triggered air-righting in rats involves the superior colliculus. Behav Brain Res 46:151-156.

Pellis SM, Pellis VC, Teitelbaum P (1991b) Air righting without the cervical righting reflex in adult rats. Behav Brain Res 45:185-188.

Rao V, Lyketsos C (2000) Neuropsychiatric sequelae of traumatic brain injury. Psychosomatics 41:95-103.

Rauch SL, Shin LM, Phelps EA (2006) Neurocircuitry models of posttraumatic stress disorder and extinction: human neuroimaging research--past, present, and future. Biological psychiatry 60:376-382.

Rauch SL, Savage CR, Alpert NM, Fischman AJ, Jenike MA (1997) The functional neuroanatomy of anxiety: a study of three disorders using positron emission tomography and symptom provocation. Biological psychiatry 42:446-452.

Riazi K, Galic MA, Kuzmiski JB, Ho W, Sharkey KA, Pittman QJ (2008) Microglial activation and TNFalpha production mediate altered CNS excitability following peripheral inflammation. Proc Natl Acad Sci U S A 105:17151-17156.

Rodgers KM, Hutchinson MR, Northcutt A, Maier SF, Watkins LR, Barth DS (2009) The cortical innate immune response increases local neuronal excitability leading to seizures. Brain 132:2478-2486.

Rolan P, Hutchinson M, Johnson K (2009) Ibudilast: a review of its pharmacology, efficacy and safety in respiratory and neurological disease. Expert Opin Pharmacother 10:2897-2904.

Rosen JB, Donley MP (2006) Animal studies of amygdala function in fear and uncertainty: relevance to human research. Biol Psychol 73:49-60.

Rothwell NJ, Strijbos PJ (1995) Cytokines in neurodegeneration and repair. Int J Dev Neurosci 13:179-185.

Rothwell NJ, Hopkins SJ (1995) Cytokines and the nervous system II: Actions and mechanisms of action. Trends Neurosci 18:130-136.

Sanberg PR, Bunsey MD, Giordano M, Norman AB (1988) The catalepsy test: its ups and downs. Behav Neurosci 102:748-759.

Sapolsky RM (2000) Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Arch Gen Psychiatry 57:925-935.

Schafers M, Sorkin L (2008) Effect of cytokines on neuronal excitability. Neurosci Lett 437:188-193.

Schallert T (2006) Behavioral tests for preclinical intervention assessment. NeuroRx 3:497-504.

Schallert T, De Ryck M, Whishaw IQ, Ramirez VD, Teitelbaum P (1979) Excessive bracing reactions and their control by atropine and L-DOPA in an animal analog of Parkinsonism. Exp Neurol 64:33-43.

Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST (2000) CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. Neuropharmacology 39:777-787.

Schmidt OI, Heyde CE, Ertel W, Stahel PF (2005) Closed head injury--an inflammatory disease? Brain research Brain research reviews 48:388-399.

Shin LM, Liberzon I (2010) The neurocircuitry of fear, stress, and anxiety disorders. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 35:169-191.

Shin LM, Rauch SL, Pitman RK (2006) Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. Annals of the New York Academy of Sciences 1071:67-79.

Shiozaki T, Hayakata T, Tasaki O, Hosotubo H, Fuijita K, Mouri T, Tajima G, Kajino K, Nakae H, Tanaka H, Shimazu T, Sugimoto H (2005) Cerebrospinal fluid concentrations of anti-inflammatory mediators in early-phase severe traumatic brain injury. Shock 23:406-410.

Simi A, Tsakiri N, Wang P, Rothwell NJ (2007) Interleukin-1 and inflammatory neurodegeneration. Biochem Soc Trans 35:1122-1126.

Simmons A, Strigo I, Matthews SC, Paulus MP, Stein MB (2006) Anticipation of aversive visual stimuli is associated with increased insula activation in anxiety-prone subjects. Biological psychiatry 60:402-409.

Sönmez U, Sönmez A, Erbil G, Tekmen I, Baykara B (2007) Neuroprotective effects of resveratrol against traumatic brain injury in immature rats. Neurosci Lett 420:133-137.

Spivak B, Shohat B, Mester R, Avraham S, Gil-Ad I, Bleich A, Valevski A, Weizman A (1997) Elevated levels of serum interleukin-1 beta in combat-related posttraumatic stress disorder. Biol Psychiatry 42:345-348.

Stein MB, Simmons AN, Feinstein JS, Paulus MP (2007) Increased amygdala and insula activation during emotion processing in anxiety-prone subjects. Am J Psychiatry 164:318-327.

Sternberg EM (1997) Neural-immune interactions in health and disease. J Clin Invest 100:2641-2647.

Sullivan RM (2004) Hemispheric asymmetry in stress processing in rat prefrontal cortex and the role of mesocortical dopamine. Stress 7:131-143.

Taupin V, Toulmond S, Serrano A, Benavides J, Zavala F (1993) Increase in IL-6, IL-1 and TNF levels in rat brain following traumatic lesion. Influence of pre- and post-traumatic treatment with Ro5 4864, a peripheral-type (p site) benzodiazepine ligand. J Neuroimmunol 42:177-185.

Thompson HJ, Lifshitz J, Marklund N, Grady MS, Graham DI, Hovda DA, McIntosh TK (2005) Lateral fluid percussion brain injury: a 15-year review and evaluation. Journal of neurotrauma 22:42-75.

Town T, Nikolic V, Tan J (2005) The microglial "activation" continuum: from innate to adaptive responses. J Neuroinflammation 2:24.

Tucker P, Ruwe WD, Masters B, Parker DE, Hossain A, Trautman RP, Wyatt DB (2004) Neuroimmune and cortisol changes in selective serotonin reuptake inhibitor and placebo treatment of chronic posttraumatic stress disorder. Biol Psychiatry 56:121-128.

Vaishnavi S, Rao V, Fann JR (2009) Neuropsychiatric problems after traumatic brain injury: unraveling the silent epidemic. Psychosomatics 50:198-205.

Vink R, O'Connor CA, Nimmo AJ, Heath DL (2003) Magnesium attenuates persistent functional deficits following diffuse traumatic brain injury in rats. Neurosci Lett 336:41-44.

von Känel R, Hepp U, Kraemer B, Traber R, Keel M, Mica L, Schnyder U (2007) Evidence for low-grade systemic proinflammatory activity in patients with posttraumatic stress disorder. Journal of psychiatric research 41:744-752.

Vyas A, Pillai AG, Chattarji S (2004) Recovery after chronic stress fails to reverse amygdaloid neuronal hypertrophy and enhanced anxiety-like behavior. Neuroscience 128:667-673.

Wagner AK, Postal BA, Darrah SD, Chen X, Khan AS (2007) Deficits in novelty exploration after controlled cortical impact. Journal of neurotrauma 24:1308-1320.

Wang F, Xu S, Shen X, Guo X, Peng Y, Yang J (2011) Spinal macrophage migration inhibitory factor is a major contributor to rodent neuropathic pain-like hypersensitivity. Anesthesiology 114:643-659.

Wheaton P, Mathias JL, Vink R (2011) Impact of pharmacological treatments on outcome in adult rodents after traumatic brain injury: a meta-analysis. J Psychopharmacol.

Willner P (1997) Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. Psychopharmacology (Berl) 134:319-329.

Woodlee MT, Asseo-Garcia AM, Zhao X, Liu SJ, Jones TA, Schallert T (2005) Testing forelimb placing "across the midline" reveals distinct, lesion-dependent patterns of recovery in rats. Exp Neurol 191:310-317.

Yagi K, Tada Y, Kitazato KT, Tamura T, Satomi J, Nagahiro S (2010) Ibudilast inhibits cerebral aneurysms by down-regulating inflammation-related molecules in the vascular wall of rats. Neurosurgery 66:551-559.

Yan F, Li S, Liu J, Zhang W, Chen C, Liu M, Xu L, Shao J, Wu H, Wang Y, Liang K, Zhao C, Lei X (2002) Incidence of senile dementia and depression in elderly population in

Xicheng District, Beijing, an epidemiologic study. Zhonghua Yi Xue Za Zhi 82:1025-1028.

Yan HQ, Banos MA, Herregodts P, Hooghe R, Hooghe-Peters EL (1992) Expression of interleukin (IL)-1 beta, IL-6 and their respective receptors in the normal rat brain and after injury. Eur J Immunol 22:2963-2971.

Yanase H, Mitani A, Kataoka K (1996) Ibudilast reduces intracellular calcium elevation induced by in vitro ischaemia in gerbil hippocampal slices. Clin Exp Pharmacol Physiol 23:317-324.

Yoshioka M, Suda N, Mori K, Ueno K, Itoh Y, Togashi H, Matsumoto M (2002) Effects of ibudilast on hippocampal long-term potentiation and passive avoidance responses in rats with transient cerebral ischemia. Pharmacol Res 45:305-311.

Yu I, Inaji M, Maeda J, Okauchi T, Nariai T, Ohno K, Higuchi M, Suhara T (2010) Glial cell-mediated deterioration and repair of the nervous system after traumatic brain injury in a rat model as assessed by positron emission tomography. J Neurotrauma 27:1463-1475.

Zhang D, Hu X, Qian L, O'Callaghan JP, Hong JS (2010) Astrogliosis in CNS pathologies: is there a role for microglia? Mol Neurobiol 41:232-241.

Zhang D, Hu X, Qian L, Wilson B, Lee C, Flood P, Langenbach R, Hong JS (2009) Prostaglandin E2 released from activated microglia enhances astrocyte proliferation in vitro. Toxicol Appl Pharmacol 238:64-70.

Figure Captions

Figure 1. Cylinder task and running wheel activity at 1 week post-injury. **(A)** LFPI rats mean number of spontaneous forelimb placements (ipsilateral and contralateral) during exploratory activity in the cylinder test did not differ from controls at 1 week post-injury. A reduction was seen in contralateral limb-use in injured rats, but this reduction did not reach significance (p=0.741). **(B)** LFPI rats mean change in distance traveled in the running wheel activity did not significantly differ from controls at 1 week post-injury. Data represent mean ± SEM.

Figure 2. Freezing behavior in a novel context. Both surgically naïve and sham operated rats froze approximately 5-10% at post-surgical measurement points before (2 weeks and 1 month) after (2 and 3 month) foot-shock (arrow). In contrast, LFPI rats froze significantly longer (~20%) than these controls before shock. After shock, untreated LFPI rats (LFPI-vehicle) nearly doubled in time freezing (~50%) whereas treated LFPI rats (LFPI+MN166) showed only a slight increase (~25%) that did not reach significance (p=0.316). The effect of injections alone (Sham+Mn166 and Sham+vehicle) were to increase freezing behavior compared to un-injected naïve and sham operated rats, particularly at the 2 and 3 month post-shock measurement points where freezing in these rats could not be distinguished from LFPI rats treated with MN166. Data represent mean ± SEM.

Figure 3. Representative images depicting GFAP immunoreactivity (reflecting astrocytic activation) assessed in the hippocampus, amygdala and insula at 3 months post-injury. LFPI rats injected with vehicle showed clear signs of reactive astrocytes (bottom row), while naïve and sham operated rats appeared to have normal astrocyte morphology. LFPI rats treated with

MN166 (third row) were difficult to differentiate from surgically naïve and sham operated groups.

Figure 4. Regional and sub-regional analyses of microglial and astroglial activation in hippocampus, amygdala and insula at 3 months post-injury. **(A-C)** LFPI vehicle injections induced a significant increase in GFAP labeling in all three regions, compared to surgically naïve, sham operated and LFPI+MN166 treated rats. **(D)** In the insula, OX-42 activation was greater in LFPI rats compared to surgically naïve, sham operated and LFPI+MN166 treated rats. There were no significant differences found between surgically naïve, sham operated and LFPI+MN166 treated rats in either analysis. Data represent mean± SEM integrated densities of immunoreactivity.

Figure Captions

Figure 1. Cylinder task and running wheel activity at 1 week post-injury. **(A)** LFPI rats mean number of spontaneous forelimb placements (ipsilateral and contralateral) during exploratory activity in the cylinder test did not differ from controls at 1 week post-injury. A reduction was seen in contralateral limb-use in injured rats, but this reduction did not reach significance (p=0.741). **(B)** LFPI rats mean change in distance traveled in the running wheel activity did not significantly differ from controls at 1 week post-injury. Data represent mean ± SEM.

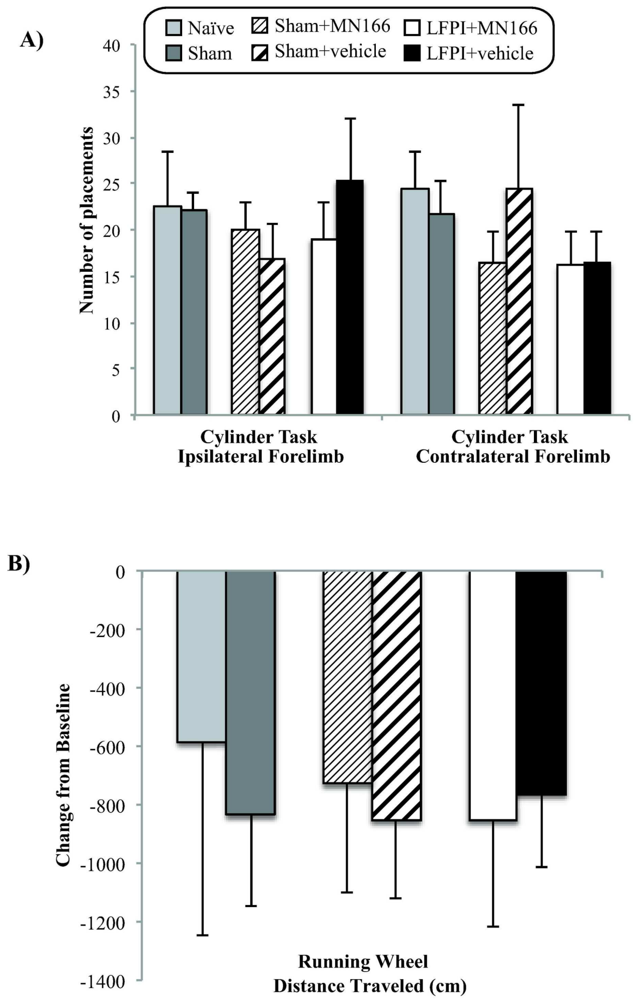
Figure 2. Freezing behavior in a novel context. Both surgically naïve and sham operated rats froze approximately 5-10% at post-surgical measurement points before (2 weeks and 1 month) after (2 and 3 month) foot-shock (arrow). In contrast, LFPI rats froze significantly longer (~20%) than these controls before shock. After shock, untreated LFPI rats (LFPI-vehicle) nearly doubled in time freezing (~50%) whereas treated LFPI rats (LFPI+MN166) showed only a slight increase (~25%) that did not reach significance (p=0.316). The effect of injections alone (Sham+Mn166 and Sham+vehicle) were to increase freezing behavior compared to un-injected naïve and sham operated rats, particularly at the 2 and 3 month post-shock measurement points where freezing in these rats could not be distinguished from LFPI rats treated with MN166. Data represent mean ± SEM.

Figure 3. Representative images depicting GFAP immunoreactivity (reflecting astrocytic activation) assessed in the hippocampus, amygdala and insula at 3 months post-injury. LFPI rats injected with vehicle showed clear signs of reactive astrocytes (bottom row), while naïve and sham operated rats appeared to have normal astrocyte morphology. LFPI rats treated with MN166 (third row) were difficult to differentiate from surgically naïve and sham operated groups.

Figure 4. Regional and sub-regional analyses of microglial and astroglial activation in hippocampus, amygdala and insula at 3 months post-injury. **(A-C)** LFPI vehicle injections induced a significant increase in GFAP

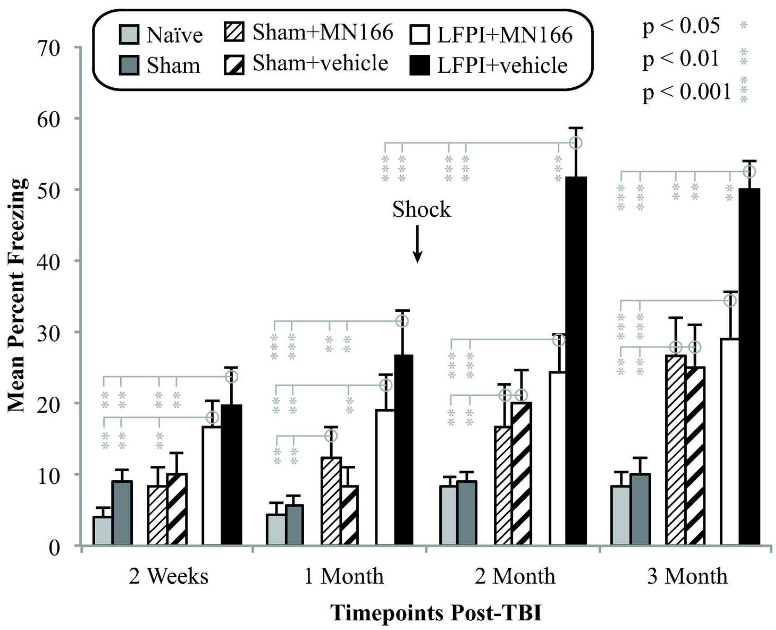
labeling in all three regions, compared to surgically naïve, sham operated and LFPI+MN166 treated rats. **(D)** In the insula, OX-42 activation was greater in LFPI rats compared to surgically naïve, sham operated and LFPI+MN166 treated rats. There were no significant differences found between surgically naïve, sham operated and LFPI+MN166 treated rats in either analysis. Data represent mean± SEM integrated densities of immunoreactivity.

Fig.1



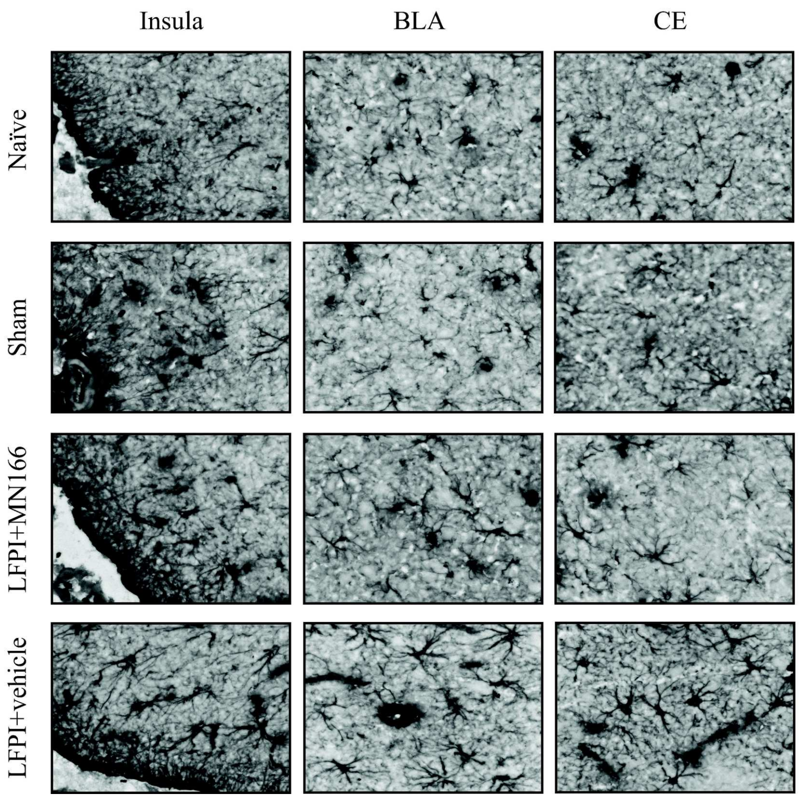
216x296mm (300 x 300 DPI)

Fig.2



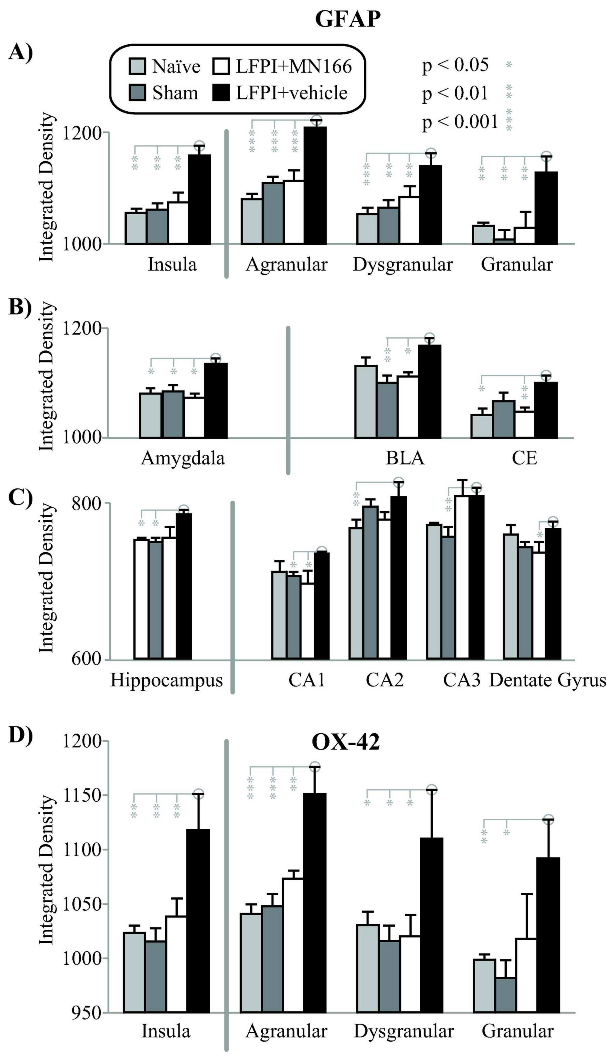
159x171mm (300 x 300 DPI)

Fig.3



175x191mm (300 x 300 DPI)

Fig.4



207x296mm (300 x 300 DPI)

Title of Study: **A Very High Speed System for Video/EEG Monitoring and Quantification of Post-traumatic Epileptogenesis**

This abstract is being submitted for (check one):

(X) Oral presentation or poster display
( ) Oral presentation only
( ) Poster display only

This presentation represents:

(X) Quantitative research ( ) Qualitative research
( ) Research utilization ( ) Combined methods
( ) Clinical innovation

Consideration for Young Investigators' Forum (check one): ( ) YES (X) NO

Research Topic: Traumatic brain injury / Healthcare informatics and medical systems

If selected, the presenter will be: Daniel Barth

A Very High Speed System for Video/EEG Monitoring and Quantification of Post-traumatic Epileptogenesis

Daniel S. Barth, Ph.D.

PURPOSE/AIMS: The overall goal of this project is to examine the role of brain inflammation in the development of post-traumatic epilepsy (PTE), and to prevent PTE with newly developed drugs that modulate the brain's immune system following injury. We use a lateral fluid percussion injury (LFPI) in the rat, a widely accepted animal model of closed head traumatic brain injury (TBI) experienced by war fighters in the battlefield. Since our major goal is to prevent the development of PTE (epileptogenesis), our first challenge was to devise methods by which we could unambiguously identify electrical (EEG) and behavioral (video) biomarkers of the epileptic brain prior to appearance of the first seizure. This is a daunting task given the vast quantities of long-term video/EEG that must be recorded and analyzed from a large number of animals.

DESIGN: To this end, during the first project year, we developed a unique system for recording, visually reviewing, and quantifying video/EEG from up to 32 animals in parallel.

POPULATION/SAMPLE STUDIED: Sprague Dawley rats with and without LFPI to the parietal and motor cortex are presently under investigation.

METHOD(S): We designed and constructed a unique recording system, based on compact amplifiers and miniature surveillance cameras, that is inexpensive, durable, and performs at a low bandwidth, permitting storage and review of large amounts of data recorded continuously for weeks.

DATA ANALYSIS: The most innovative component of the system is our specially designed software that permits very fast and interactive visual examination and event identification of hours of video/EEG data in minutes. The driving principle behind this software is that the human visual system is far more skilled and reliable than automated systems for identifying epileptiform events in the EEG and validating these events with video-recorded behavior. The core of our software is a graphical user interface that makes this possible for minimally trained users.

FINDINGS: Our system is now allowing us to precisely identify potential EEG biomarkers during epileptogenesis, when intervention may be possible. The system is also allowing us to statistically quantify both abnormal and normal EEG activity from long-term recording in animal models of TBI.

CONCLUSIONS/RECOMMENDATIONS: It is now possible to record and visually analyze long-term video/EEG data following brain trauma at a minimum cost and with sufficient speed and accuracy to make statistical analysis of normal and pathological EEG biomarkers possible for the first time.

IMPLICATIONS: We have begun analyzing epileptogenesis in LFPI and in a pilocarpine model. We have identified archetypical patterns in the normal EEG that could be easily mistaken for epileptiform. This knowledge will serve as critical foundation for studying the effects of neuro-immune modulating compounds following trauma in preventing epileptogenesis during the second project year. We also anticipate an unplanned application to military medicine to detect potential post-traumatic neurological disturbance and facilitate return to duty decisions.

FROM/TO TIME PERIOD OF STUDY: 07/01/11 to 06/30/12

FUNDING: CDMRP #PR100040

For presentation at SFN 2013

Hippocampal auditory evoked potentials in conjunction with continuous long-term video-EEG monitoring reveals novel biomarkers for epileptogenesis in the lithium-pilocarpine model of epilepsy in rats.

Acquired temporal lobe epilepsy in humans and in animal models has typically been characterized by an initiating traumatic event (brain trauma, status epilepticus, etc.) followed by a latent or "silent" period lasting weeks to many months during which epileptogenesis is presumed to occur; a period terminated by the appearance of spontaneous recurrent seizures (SRS) and the establishment of chronic epilepsy. Yet, there has been recent evidence suggesting that, while the latent period is behaviorally silent, there may be electrographic changes both spontaneous and evoked that reflect neural circuit and cellular alterations underlying epileptogenesis, and that epileptogenesis may continue well past presentation of initial SRS.

The objective of the present study was to compare biomarkers of epileptogenesis in the lithium-pilocarpine (status epilepticus; SE) rat model using regular (2 epochs/hr) hippocampal auditory evoked potentials (HAEP) to probe excitability changes, continuous spectral analysis of hippocampal and cortical EEG, automated epileptiform spike density (ESD) analysis, and continuous movement analysis based on video optical flow. Of these measures, HEAP were by far the most sensitive and reliable biomarker, showing marked and stereotyped alterations in morphology and amplitude during the latent period. Interestingly, HEAP morphology continued to change weeks or even months after the animals began having SRS, suggesting that epileptogenesis continued well after the latent period. In addition there was a circadian rhythm to the amplitude of HEAP, as well as EEG delta/theta power, and movement, with a higher probability for seizures during the late dark phase accompanying HEAP suppression. These results indicate that chronic HEAP recording may be a useful tool for actively probing the hippocampal system in order to reveal changes in its underlying physiology accompanying epileptogenesis during the latent and post-latent periods.

Research Idea: This proposal directly addresses two FY13 ARP Areas of Interest. We will examine conditions co-occurring with ASD (examining neuroinflammation in our newly developed animal model of comorbid ASD and epilepsy, **AIM 1**) and validate new or existing therapeutic targets (using anti-inflammatory micro-glia suppressant compounds, that are safe for eventual translation to human use, to prevent limbic hyper-excitability associated with ASD/epilepsy, **AIM 2**).

How the research addresses a central problem in ASD: The close association between ASD and epilepsy has been recognized for decades [1,2]. Early investigations of electroencephalographic (EEG) abnormalities in autistic children spearheaded the concept of ASD as a neurological as opposed to purely psychiatric disorder [2,3], and subsequently established the epileptiform characteristics of these abnormalities, revealing a remarkably comorbid syndrome. The link between ASD and epilepsy may hold important clues to the etiology of both disorders, and suggests that in some cases they may share a common neurological basis. While genetics no doubt play a substantial predisposing role [4,5], there are converging lines of evidence that the maternal environment during pregnancy may also be of paramount importance; prenatal exposure to specific environmental factors markedly increases risk, and may establish a dynamic neuropathy resulting in autistic behavior and seizures. Little can be done to alter genetic predisposition, however, a better understanding of prenatal environmental factors could lead to improved strategies for decreasing risk and for intervention.

Ideas and reasoning on which the proposed project is based: The neurobiological basis for ASD remains largely unknown, but recent research strongly supports contributions of genetic, environmental, neurological, *and immunological* factors [6-10]. Immune dysfunction is a remarkably consistent finding in ASD, proposed as a critical mechanism for ASD pathogenesis [11]. Yet, most studies note abnormal elevations of blood-born immune activation markers. Only recently have neuroinflammatory processes also been described in the cerebral cortex and white matter in ASD [9]. Neuroinflammation in ASD is characterized by marked "reactive gliosis", where astrocytes, and particularly microglia cells, release proinflammatory cytokines as part of the innate immune response to stress, toxins, infection, and apoptosis. This finding is pivotal because these cytokines are known to be massively excitatory to neurons, potentially triggering excitotoxic cascades in the developing brain that could result in abnormal development <u>and</u> in *chronic hyperexcitability* [12]. Remarkably, 30% of children with ASD develop chronic epilepsy and 50-90% show sub-clinical epileptiform spikes by adolescence [13-16]. Similarly, approximately 30% of children with epilepsy are also diagnosed with ASD [17]. The role of glial cytokines in *epilepsy* is under intense investigation [18].

Preliminary Data: Our laboratory has recently completed two studies through a seed grant funded by Autism Speaks Foundation and one study concerning post-traumatic epilepsy funded by DoD, providing data leading to this idea development proposal. First, we discovered that bacterial activation of microglia (induced by lipopolysaccharide; LPS) produces seizures [19] <u>and microglia suppressant drugs inhibit development of epilepsy</u> in a lithium/pilocarpine model. Thus, neuroinflammation can induce seizures and immuno-modulation has anti-epileptogenic properties. Second, we replicated findings of Zerrate and colleagues [20] using the known ASD teratogen, terbutaline (used to arrest pre-term labor and associated with increased concordance for autism in dizygotic twins), to produce ASD-like behavior in rats and discovered <u>microglia suppressant drugs prevent development of these behaviors,</u> again suggesting the role of neuroinflammation in ASD and immuno-modulation as a potential therapeutic target. Finally, we have recently succeeded in developing an animal model of <u>ASD *and* epilepsy</u>. We discovered that <u>maternal stress combined with terbutaline</u> results in ASD like behavior more severe than either treatment alone, and <u>only the combination</u> results in development of chronic epilepsy. This animal model of ASD/epilepsy will serve as an ideal platform for testing the following hypotheses.

Hypotheses: Given, A) the close association between ASD and immune dysfunction, B) recent evidence for elevated innate immune responses and proinflammatory cytokine levels in the ASD cortex, C) epidemiological linkage between ASD, brain hyperexcitability, and epilepsy, D) strong evidence linking microglia activation with increased brain excitability and seizures and E) our recent discovery that combining two known neuroinflammatory teratogens results in ASD-like behaviors and development of chronic spontaneous recurrent seizures (the hallmark of epilepsy), <u>we hypothesize that a core mechanism underlying both pre- and postnatal neuro-developmental disorders in ASD is chronic neuroimmune induced hyperexcitability. If true this would predict that small molecule microglia suppressant drugs, capable of crossing into the brain, would suppress release of inflammatory cytokines and attenuate pre- and postnatal hyperexcitability and neurodegeneration</u> in our maternal stress + terbutaline model of ASD/epilepsy.

Specific Aims:

Aim 1) Develop the maternal stress + terbutaline model of ASD/Epilepsy and establish baseline biomarkers for neuroinflammation, ASD–like behavior, and epileptogenesis.

Aim 2) Examine the effectiveness of acute glial suppression on behavioral, functional, and histological end-points established in Aim 1, with the objective of demonstrating effectiveness in preventing or attenuating ASD/epilepsy development.

Research Plan:

Aim 1) Four groups of 5 pregnant rats will be obtained at embryonic day two (E2). Dams will be randomly assigned to two stressed and two non-stressed groups. Prenatal stress will be administered daily from (E4) until delivery. On (E4), (E11) and (E18) dams will be placed in a fear conditioning chamber and shocked (0.5 mA, 2 sec duration, delivered at 60 and 120 sec) and re-exposed daily to the fear-conditioning chamber for 5 min. Non-stressed dams will be left undisturbed in their home cages. At birth, male pups from each group will be randomized and redistributed, allowing each dam to foster no more than one pup from their own litter. Pups from entire litters will be divided into 4 experimental groups, 1 maternally stressed and 1 unstressed group receiving subcutaneous injections of terbutaline hemisulfate (10 mg/kg) and the other stressed and unstressed groups receiving equivalent volumes of saline (1 ml/kg) on postnatal day (PN) 2 through 5. Pups will be maintained until adolescence (2 mo), receive behavioral tests (ultrasonic vocalizations, freezing in a novel environment, open field social interaction, acoustic pre-pulse inhibition, exploratory behavior, locomotor and repetitive/stereotypic activity). Pups will then be implanted with one SS screw electrode over primary auditory cortex and one bipolar electrode in dorsal hippocampus and attached to a head mount for chronic tethered video/EEG recording. Along with EEG, averaged auditory evoked potentials (AEP) will be recorded every 30 minutes. Continuous recordings will be performed for 3 months. Animals will then be sacrificed and immuno-reactivity assessed with markers of microglia (OX-42) and astrocytes (GFAP). Video/EEG recordings will be analyzed for seizures and epileptiform spiking using custom software written by the P.I. and already in use in our laboratory. AEP amplitude and morphology changes will also be tracked to probe for changes in brain excitability (similar to systematic AEP waveform changes we have noted for our pilocarpine epilepsy model during the development of chronic epilepsy). Between group comparisons of long-term differences in activity level (our preliminary studies showed marked hyperactivity in autistic rats) will also be performed using optical flow analysis of the video records. Aim 1 will provide foundational baseline biomarkers of ASD/epilepsy for comparison to treatment groups in Aim 2. We expect it will require 1 year to complete Aim 1.

Aim 2) Procedures for Aim 2 will be the same as Aim 1, except that 4 additional groups will be included, receiving daily s.c. injections of MN166 (Ibudilast) or vehicle alone for a 5 day period following the last terbutaline/saline injection. MN166 is a well-documented cyclic AMP phosphodiesterase inhibitor that crosses the blood brain barrier and effectively suppresses microglia activated cytokine release by antagonizing TLR4 receptors [21]. We have used this acute dosing regime in our preliminary studies to effectively to block development of ASD-like behavior in the terbutaline (alone) model and also to interrupt epileptogenesis in the lithium/pilocarpine model. We expect MN166 to effectively block or at least attenuate development of ASD-like behavior and epilepsy in Aim 2. We expect it will require 2 years to complete Aim 2.

Impact: The ideas developed here should have a major scientific impact on the CNS mechanisms of ASD by linking, for the first time, innate neuro-immune inflammation to potentially epileptiform hyperexcitability and degenerative neuro-development. It has been established in recent epilepsy research that innate immune responses lower thresholds for seizures and that epileptiform hyperexcitability induces innate immune responses and inflammatory cytokine release, <u>establishing a potentially destructive positive feedback loop for chronic epileptogenesis</u>. We expect the results of the ASD/epilepsy model developed here to provide an essential bridge between positive results in epileptology to our understanding of ASD pathophysiology. While the proposed work has some risk, our expectations are backed by promising preliminary data in both the autism and epilepsy fields. Success here could have a very rapid impact on outcomes of individuals with ASD. While there are several immuno-suppressant anti-inflammatory drugs we could have chosen to evaluate, we chose MN166 because it can be orally administered, crosses the blood-brain barrier, and has no known detrimental side effects in humans (it has been used in Asia for years to treat bronchial asthma). MN166 is also currently under FDA clinical trials for treatment of chronic pain in humans [21,22]. Finally, we chose MN166 because we have preliminary indications of a powerful preventive effect in the development of post-traumatic epilepsy in the rat. If successful, we expect this work to be rapidly translatable to use as a potential drug for prenatal prevention of ASD and/or for postnatal prevention of associated epilepsy, potential behavioral interference due to hyperexcitability, and continued developmental regression from excitotoxicity in the immunoreactive ASD brain.

Innovation: To our knowledge, this is the first and only ASD - epilepsy - neuroinflammation animal model to be developed. We are combining ASD/epilepsy, ASD/innate immunity, and epilepsy/innate immunity, all very active but, until now, separate areas of research. Based on our preliminary work in post-traumatic epilepsy prevention, we are specifically targeting microglial suppression as a unique path for ASD prevention and/or treatment, which, if successful, will present a completely novel and we think extremely productive paradigm for ASD research. We also add novel biomarkers (AEP and Optical Flow Movement analysis) to other behavioral and electrophysiological measures to monitor the progress of brain excitability and treatment effects. Finally, this work represents a unique combination of electrophysiological, behavioral, histological, (Barth) and immunological (Watkins) methodologies for examining mechanisms and prevention in a novel animal model of ASD/epilepsy.

Statement of Work (SOW)

Task (specific aim) 1. Test the efficacy of proximal (within 24 hr. of injury) MN166 treatment on development of post-traumatic anxiety: **(timeframe, months 1-22)**

1a. Animal use approval for all experiments. University of Colorado Institutional Animal Care and Use Committee. **(timeframe, months 1-2)**
1b. Perform preliminary studies of lateral fluid percussion injury (LFPI) impact pressures required to reliably produce mild and severe TBI and associated post-traumatic anxiety. **(timeframe, months 3-7)**
Assay-> Behavioral measures of post-traumatic anxiety (freezing in novel environment, open field and elevated plus exploration) will have been quantified for mild and severe TBI and statistically compared to our existing LFPI model of moderate TBI.
1c. Perform motor and behavioral testing of 8 groups (10 rats each: Sham-operated/vehicle-injected, Sham-operated*/MN166-injected*, mildTBI*/vehicle-injected*, mildTBI/MN166-injected, moderateTBI*/vehicle-injected, moderateTBI/MN166-injected, severeTBI*/vehicle-injected, severeTBI/MN166-injected) receiving MN166 treatment at 24 hr. post-injury. **(timeframe, months 8-13)**
Assay-> Motor testing will have been performed prior to and following Sham-Operated or TBI, along with behavioral testing before foot-shock and after foot-shock at 2 weeks, 1, 2, 3 and 6 month intervals.
1d. Prepare and submit progress report #1 to DoD. **(timeframe, month 12)**
1e. Perform immunohistochemistry of all experimental groups. **(timeframe, months 14-18)**
Assay-> Densitometry will have been performed on GFAP and OX-42 stained sections of all rats.
1f. Statistically analyze and prepare graphical representations of results. **(timeframe, months 19-20)**
Assay-> Analyzed data from sub-aims 1c and 1e shall have been converted to figures for publication.
1g. Submit and revise manuscript for publication. **(timeframe, months 21-22)**
Assay-> A manuscript describing results for task 1 shall be finished and submitted for publication in a peer refereed scientific journal.

Milestone #1: Demonstration that early treatment with MN166 prevents or attenuates post-traumatic anxiety in mild, moderate, and perhaps severe TBI.

Task (specific aim) 2. Test the efficacy of delayed (1 mo. post-injury) MN166 treatment on existing post-traumatic anxiety: **(timeframe, months 23-36)**

2a. Perform motor and behavioral testing of 8 groups (10 rats each: Sham-operated/vehicle-injected, Sham-operated*/MN166-injected*, mildTBI*/vehicle-injected*, mildTBI/MN166-injected, moderateTBI*/vehicle-injected, moderateTBI/MN166-injected, severeTBI*/vehicle-injected, severeTBI/MN166-injected) receiving MN166 treatment at 1 mo. post-injury. **(timeframe, months 23-28)**
Assay-> Motor testing will have been performed prior to and following Sham-Operated or TBI, along with behavioral testing before foot-shock and after foot-shock at 1-month through 6-month intervals.
2b. Prepare and submit progress report #2 to DoD. **(timeframe, month 24)**
2c. Perform immunohistochemistry of all experimental groups. **(timeframe, months 29-32)**
Assay-> Densitometry will have been performed on GFAP and OX-42 stained sections of all rats.
2d. Statistically analyze and prepare graphical representations of results. **(timeframe, months 33-34)**
Assay-> Analyzed data from sub-aims 1c and 1d shall have been converted to figures for publication.
2e. Submit and revise manuscript for publication. **(timeframe, months 35-36)**
Assay-> A manuscript describing results for task 1 shall be finished and submitted for publication in a peer refereed scientific journal.
2f. Prepare and submit final progress report to DoD. **(timeframe, month 36)**

Milestone #2: Demonstration that delayed treatment with MN166 reverses or attenuates established post-traumatic anxiety in mild, moderate, and perhaps severe TBI.

1) BACKGROUND

Post-traumatic anxiety is a leading and devastating consequence of human traumatic brain injury (TBI). Traumatic brain injury (TBI) has been described as the signature injury of the wars in the Middle East, where improvised explosive devices, suicide bomb blasts, and other combat related head trauma have seen a marked increase. This precipitous rise is highly correlated with a substantial increase of war fighters suffering from chronic post-traumatic stress disorder (PTSD), of which post-traumatic anxiety is the dominant symptom. In the general public, TBI is also a rising health concern, with approximately 1.7 million people in the United States alone sustaining a TBI each year and more than 5.3 million living with TBI-related disabilities. In addition to physical, cognitive and behavioral impairments, the long-term consequences of TBI include increased risk of neuropsychiatric disorders, of which anxiety disorders are by far the most prevalent, with rates ranging from 10-70% across studies (1, 2).

There is a lack of effective treatment for post-traumatic anxiety. Clinical trials to date have failed to reveal an effective treatment for post-traumatic anxiety. This is due in part to the considerable overlap in symptoms and high rates of co-occurrence between TBI and anxiety disorders (3-7). Co-morbid TBI/Anxiety has only been recently acknowledged as a clinical syndrome and there is a lack of research-based evidence addressing pharmacological approaches to treatment, as studies focusing on affective disorders typically exclude patients with a history of TBI and vice versa (6). The 3 primary pharmacological agents used to treat anxiety (anti-depressants, anticonvulsants, and anxiolytics) all lack evidence for treating co-morbid TBI/Anxiety (6, 8).

The neurophysiology of post-traumatic anxiety is poorly understood. Failure to develop adequate treatment for post-traumatic anxiety is also due to poor understanding of its neurophysiological basis. Studies of the etiology of anxiety disorders implicate exaggerated responses of the amygdala and insula (9-13), impaired inhibition of medial prefrontal cortex and anterior cingulate (12, 14-16) and decreased hippocampal volume (15, 17, 18). Several studies have shown greater activation of bilateral amygdala and insula in patients with a variety of anxiety disorders, including obsessive/compulsive disorder, phobia and post-traumatic stress disorder (PTSD) (9-13). Specifically related to PTSD, magnetic resonance imaging (MRI) studies have reported structural, neurochemical and functional abnormalities of medial prefrontal cortex in patients with PTSD, including anterior cingulate cortex (12, 15). Functional neuroimaging has also shown diminished responses in medial prefrontal cortex, which is thought to play a role in an array of anxiety disorders. Neuroimaging studies of the hippocampus have found decreased volumes in patients with PTSD, which is thought to be a risk factor for the development of pathological stress responses (15, 17, 18). Yet, in spite of substantial human neuroimaging reports, the *cellular mechanisms* potentially leading to TBI-induced neurochemical, anatomical, and functional abnormality in these structures are poorly understood.

Neuroimmune inflammation may play a key role in post-traumatic anxiety and as been largely ignored in developing treatments. Extensive literature indicates that inflammation produced by neuroimmune responses to TBI could be fundamental to the neurophysiology of post-traumatic anxiety (19-24). It is well established that post-traumatic inflammatory mediators activate microglia and astrocytes during the innate immune response to injury, leading to the expression of high levels of proinflammatory cytokines, most notably interleukin 1 beta, “IL-1β”, interleukin 6, “IL-6”, and tumor necrosis factor-α, “TNF-α” (22, 25-28). Given that these cytokines participate in autonomic, neuroendocrine and behavioral responses to brain injury, destabilize neurotransmitter release and re-uptake, negatively impact neuronal integrity and survival, and initiate neurotoxic processes, they may contribute to functional alterations of brain areas involved in post-traumatic anxiety (29-31).

Glial activation is normally neuroprotective (26, 32); however, the *chronic* inflammatory responses and exaggerated proinflammatory cytokine levels observed following injury initiate neurotoxic processes resulting in secondary tissue damage (20, 33-35), neuronal death (29, 36-38), secondary injury cascades (39-43) and neuronal hyperexcitability (28, 34, 38, 44). There is substantial support for chronic inflammation following TBI.

Both human and animal studies have shown that neuroinflammation is an ongoing process that persists for months to years following the injury (45-48). The ongoing inflammatory response to tissue injury may contribute to damage and dysfunction in brain regions associated with anxiety, as TBI has been found to induce both acute and chronic neurodegeneration that could be caused by delayed cellular death pathways initiated by complex signaling cascades in activated glial cells (49, 50). Several new lines of evidence support this hypothesis: (a) innate immune responses triggered by TBI (20, 34, 35), (b) resultant prolonged post-traumatic release of proinflammatory cytokines by activated glial cells (29, 33, 51, 52), (c) chronic peripheral elevations of proinflammatory cytokines in patients with PTSD and panic disorder (19, 21, 23, 24), (d) increased levels of activated microglia and astrocytes, IL-1β, TNF-α and IL-6 following controlled cortical impact and weight drop injury in rats (53-56), (e) increased anxiety-like behavior (57-60), and (f) elevated plasma corticosterone concentrations (61) when these cytokines are administered centrally or systemically in rats. Overall, these findings provide evidence for a potential role of the neuroimmune system in the pathophysiology of post-traumatic anxiety. Our over-riding hypothesis is that suppression of injury-induced glial cell activation may have eventual promise for attenuation of the development of post-traumatic anxiety *or treatment of existing post-traumatic anxiety* in humans, a hypothesis supported by our preliminary data indicating a powerful prophylactic effect of glial suppressant drugs on development of post-traumatic anxiety and the ability to reverse post-traumatic anxiety in an animal model we have developed.

Animal models of post-traumatic anxiety have only very recently been developed to study mechanisms and interventions. In light of the high prevalence and clinical impact of post-traumatic anxiety in human TBI and our poor understanding of its mechanisms and treatment, it is surprising that psychiatric sequelae of brain trauma have been largely overlooked in animal research, which has focused instead on sensory/motor and cognitive deficit models (62). However, there have been recent seminal reports by Vink and colleagues (63-65) of post-traumatic anxiety-like behavior in rats using an impact-acceleration model of diffuse TBI and noting significant decreases in open field exploration due to freezing (a natural defensive fear response of rats). Several other groups have since reported similar post-traumatic anxiety in rodents using a variety of TBI methods, post-injury measurement time-points, and behavioral measures (66-70). Freezing behavior was not measured directly in these studies, although freezing was consistently reported to cause decreased exploratory behavior in the open field test (63-65), suggesting a dominant fear response that is associated with pathological anxiety. In none of studies could freezing be attributed to injury induced motor deficits since there are only transient (~ 1 week) motor deficits following fluid percussion (69, 73-76) as well as controlled cortical impact (67, 70, 77-83) injuries in the rat. Particularly relevant to the present proposal concerned with animal models of post-traumatic anxiety resulting from battlefield brain trauma, is the recent work by Ahler's group at the Operational and Undersea Medicine Directorate Naval Medical Research Center (71). Independent of our recently published results of post-traumatic anxiety induced by lateral fluid percussion injury (LFPI) in rats (72), they discovered that blast exposure in rats results in increased anxiety and enhanced contextual fear conditioning, both of which are PTSD-related behavioral traits. These findings are exciting because, similar to our work, brain trauma was induced during anesthesia, indicating a purely physiological component to resulting post-traumatic anxiety (as opposed to confounds introduced by additional psychological trauma during injury). Furthermore, the physiological basis of the post-traumatic anxiety in this study appeared to predominantly involve the amygdala (we have found involvement of amygdala, insula and hippocampus in our work). Thus, two distinct animal models of post-traumatic anxiety, blast injury and LFPI, have independently been shown to result in very similar behavioral and physiological symptoms, greatly increasing our confidence in the validity, reproducibility and generalizability of the animal work in this field.

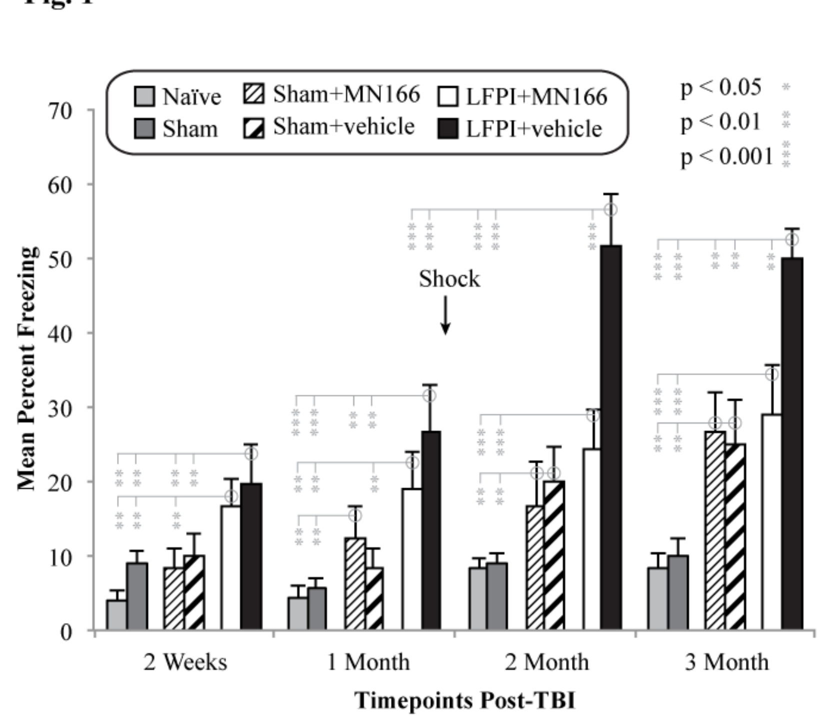
Preliminary Work

As mentioned above, we have recently developed a rodent model of post-traumatic anxiety (84) that directly measures freezing in a novel environment as the *most* reliable index of abnormal/pathological anxiety induced by LFPI. Other measures of anxiety, such as open field and elevated plus maze exploration, showed results that trended in the same direction but did not reach significance with the number of rats examined in these studies. With this model of LFPI induced freezing behavior, we have collected compelling preliminary data indicating a major role of glial activation in insula, amygdala and hippocampus in the development of post-traumatic anxiety.

In this preliminary work we attenuated the proinflammatory response of glial cells to LFPI using a well documented, cyclic AMP phosphodiesterase inhibitor, MN166 (“Ibudilast”; 3-isobutyryl-2-isopropylpyrazolo-[1,5-a]pyridine). MN166 has a documented suppression of pro-inflammatory responses by microglia *in vitro* and *in vivo*, tolerability, and long duration of action (64). Systemic or central administration attenuates nerve injury-induced astrocytic and microglial activation and also suppresses expression of the cytokines IL-1β, IL-6 and TNFα *in vivo*. MN166 partitions roughly 1:1 plasma:brain in rats (65) with a half-life nearly the same in both plasma and CNS tissues ~1hr after a single injection and ~8hr following a multi-day regimen wherein the drug has reached steady state in both compartments (65, 66). It attenuates the activation of brain microglia following *in vivo* administration in the rat (64, 67). At concentrations of 10 mg/kg, onset of glial attenuation with MN166 in rats is within 24 hr. and durable for >24 hr. (64, 65, 68-70). Interestingly it has recently been shown that MN166 administration can increase the anti-inflammatory cytokine IL-10 expression close to 40 fold in vivo (71). It is therefore likely that much of the glial inhibitory actions of MN166 are a result of the MN166 mediated increase in IL-10 release. The only known effects of MN166 on neurons are indirect via glial actions (72). Based on clear evidence from animal studies indicating a role of glial cells in pathological pain states, MN166 is currently under the FDA approved Phase 2 clinical trials in the treatment of neuropathic pain. It was chosen for use in our preliminary studies (and in the present proposal) because it is an orally available, blood-brain barrier permeable, glial activation inhibitor (based on microglial and astrocyte activation marker suppression). MN166 also has a long history of safety in humans and has been used widely for over 15 years in Japan to treat post-stroke dizziness and asthma (72). Finally, recent controlled studies indicate that it is well tolerated in healthy adults (73). Thus, potential future translation of this compound for eventual use in human post-traumatic anxiety is not as distant as other unexplored compounds.

A major outcome of our preliminary work is that we discovered peri-injury application of MN166 has the powerful effect of attenuating post-traumatic glial activation (in amygdala and insular cortex, brain regions closely associated with anxiety in humans and animals) and preventing development of post-traumatic anxiety-like behavioral symptoms. Additionally, our data indicate that ongoing post-traumatic anxiety-like behavior may be due to a chronic inflammatory response that can be effectively reversed by a brief (5-day) treatment of MN166 *once anxiety-like symptoms have fully developed at 1 month post-injury*. Our key results are summarized below.

Fig. 1

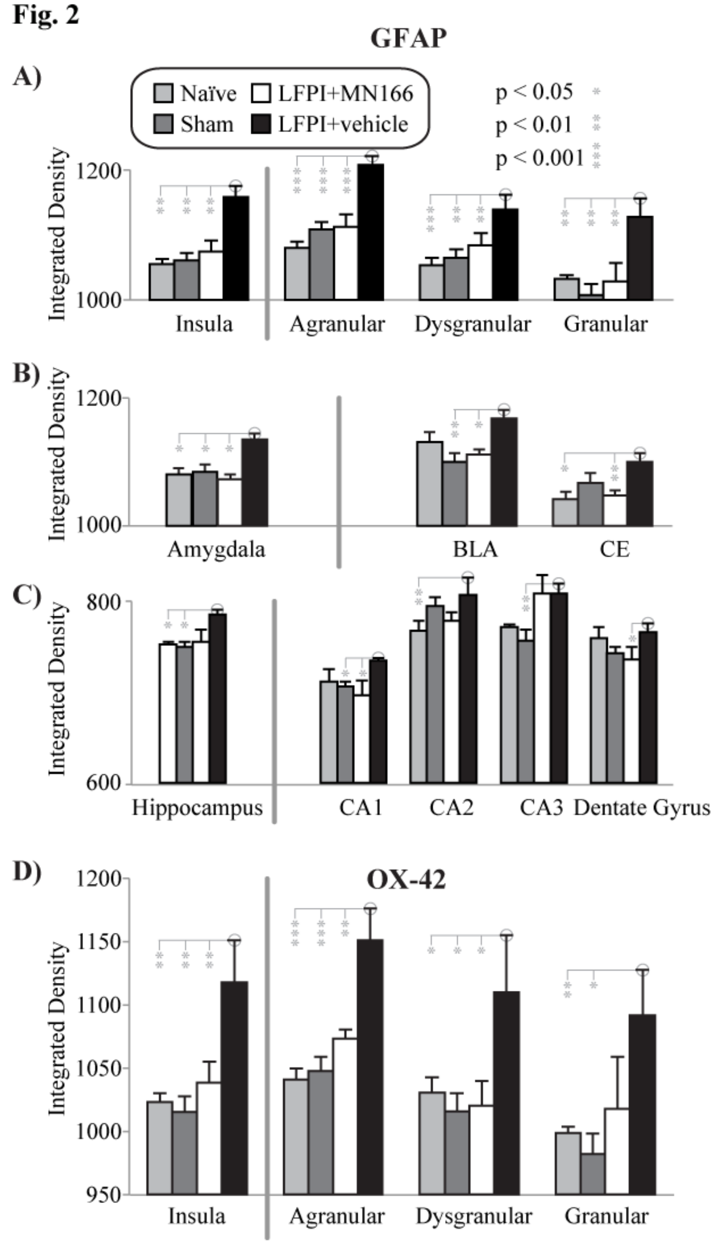


(Rodgers, et. al., 2012)

Preliminary Study 1: Evidence for prevention of post-traumatic anxiety with peri-injury glial attenuation

Glial attenuation reduces freezing behavior in the novel context. Our initial study focused on the effectiveness of attenuating injury-induced neuroinflammation on preventing the development of post-traumatic anxiety, using daily MN166 injections beginning 1 day before LFPI, the day of LFPI, and continuing for 3 days following injury. The justification for beginning injections before injury was based on the approximately 24 hr. required for MN166 to maximally attenuate glial activation. Despite normal motor, vestibular and locomotive function, LFPI produced large increases in freezing behavior when rats were placed in a novel context (**Fig. 1**). Exposed only to this minor stressor (i.e. at 2 week and 1 month post-injury measurements conducted prior to shock), LPFI rats injected with either MN166 or vehicle (**Fig. 1**; white and black bars, respectively) froze approximately twice as long as naïve or sham operated rats (**Fig. 1**; light and dark grey bars, respectively). At 2- and 3-month measurement times, following the additional major stressor of shock (**Fig. 1**; arrows), freezing in the novel context (testing was not performed in the shock chamber to avoid confounds of conditioned fear) in both naïve and sham operated rats remained constant at approximately 10%. Freezing in LFPI rats treated with MN166 remained consistently higher than these controls, but, while appearing higher compared to earlier post-injury measurements in the same animals, this increased freezing compared to naïve and sham operated rats before (1 month) and following (2 month) shock did not reach significance. By contrast, LFPI+vehicle rats nearly doubled their freezing time to approximately 50% (**Fig. 1**; black bars) compared to pre-shock values, freezing approximately twice as long as LFPI+MN166 rats and 5 times as long as naïve and sham operated controls at the 2- and 3-month post-injury measurement times. The behavioral effects of injections alone, independent of LFPI, are reflected in sham surgery groups with injections of either MN166 or vehicle (**Fig. 1**; narrow and broad diagonal lines, respectively). Sham operated rats tended to freeze more than un-injected naïve and sham operated controls, reaching significance for both groups at the 2- and 3-month measurement points and suggesting that injections alone are aversive and can contribute to subsequent freezing. However, even at pre-shock measurement points, LFPI animals that received the same injections of MN166 or vehicle froze significantly more than injected controls, indicating substantial enhancement of freezing produced by LFPI. This effect became more apparent following shock, where LFPI+vehicle rats froze twice as long as the injected controls. By contrast, LFPI+MN166 rats were not distinguishable from either injected control group following shock, suggesting that their elevated freezing compared to naïve and sham operated animals was the result of injections alone and that MN166 eliminated the exaggerated freezing response to shock characterizing LFPI+vehicle rats.

Fig. 2



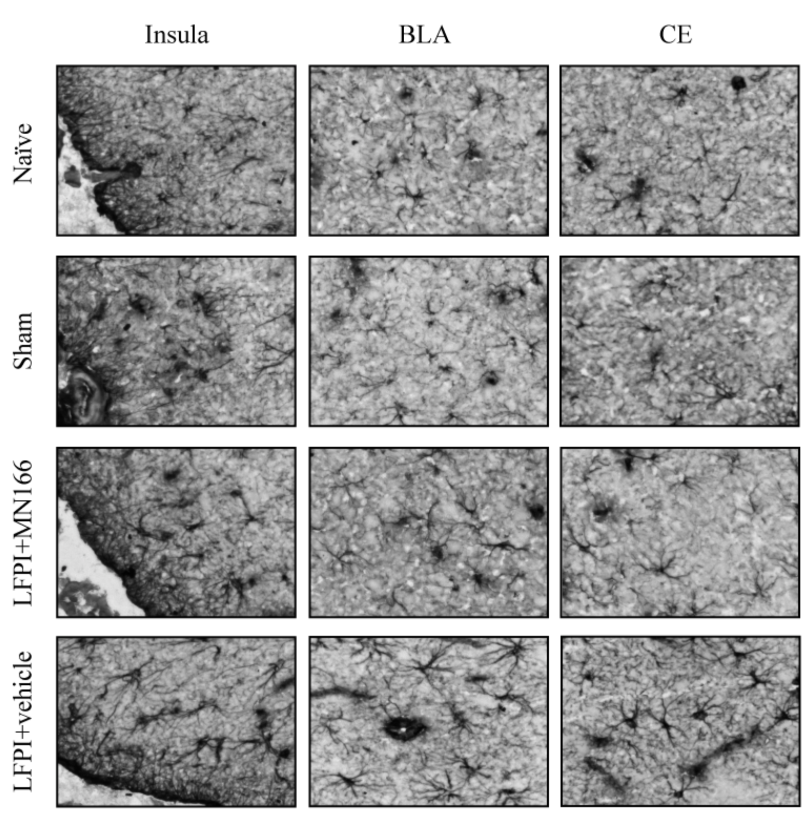
(Rodgers, et. al., 2012)

Reactive gliosis in hippocampus, amygdala and insula at 3 months post-injury. Consistent with our behavioral results suggesting a role for LFPI induced neuroinflammation in post-traumatic anxiety, LFPI rats with only vehicle injections (**Fig. 2**; black bars) displayed a pattern of increased glial fibrillary acidic protein (GFAP), indicating reactive astrocytes in insula (**Fig. 2A**), amygdala (**Fig. 2B**) and hippocampus (**Fig. 2C**). By contrast, GFAP labeling in LFPI rats receiving MN166 injections (**Fig. 2**; white bars) were not distinguishable from surgically naïve (**Fig. 2**; light grey bars) or sham operated (**Fig. 2**; dark grey bars) animals, indicating a significant reduction in injury-induced astrogliosis due to MN166 treatment. OX-42 (a microglial marker) activation was also greater in the insular cortex of LFPI rats compared to surgically naïve, sham operated and

LFPI+MN166 treated animals (**Fig. 2D**). There were no significant differences found between surgically naïve, sham operated and LFPI+MN166 treated rats in either analysis.

Astrocyte morphology 3 months post-injury. **Figure 3** shows representative images depicting GFAP immunoreactivity assessed in the hippocampus, amygdala and insula at 3 months post-injury. LFPI rats injected with vehicle showed clear signs of reactive astrocytes (bottom row), while naïve and sham operated rats appeared to have normal astrocyte morphology. LFPI rats treated with MN166 (third row) were difficult to differentiate from surgically naïve and sham operated groups.

Fig. 3

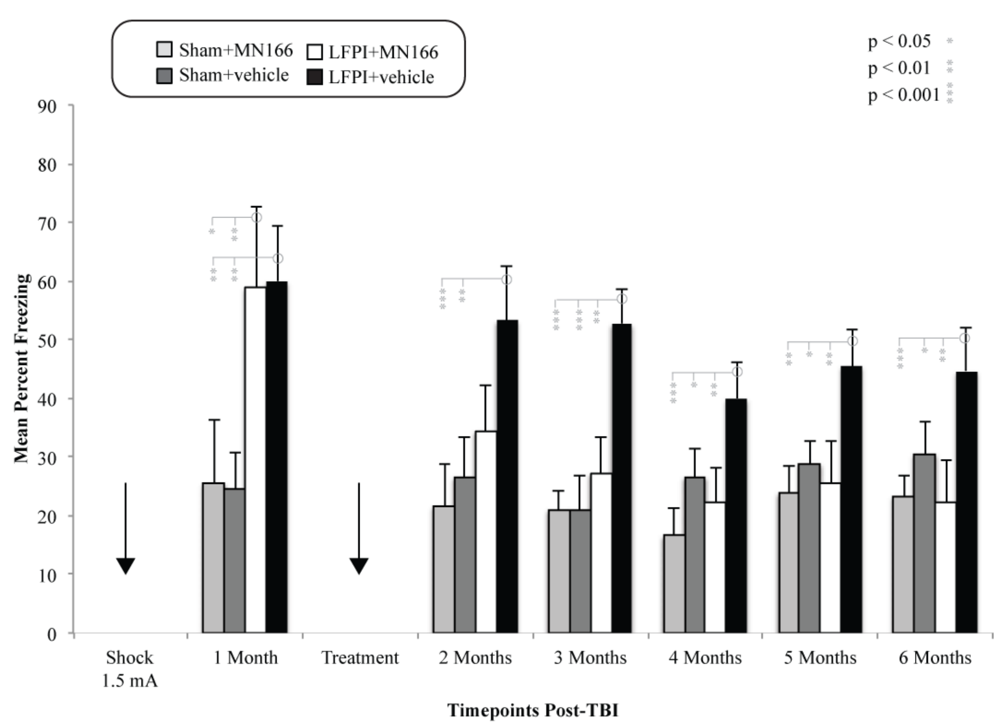


(Rodgers, et. al., 2012)

Preliminary Study 2: Evidence for treatment of post-traumatic anxiety with post-injury glial attenuation

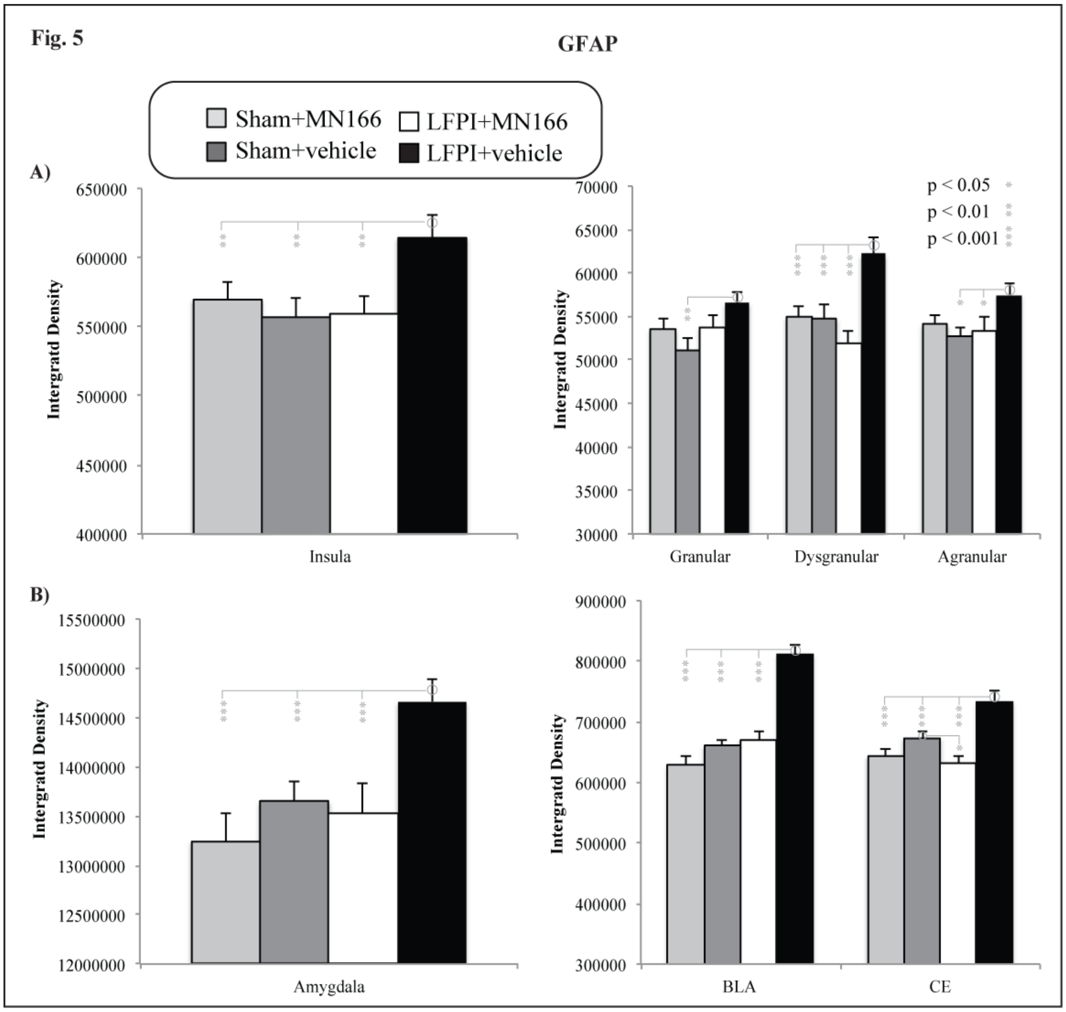
Glial attenuation reduces established freezing behavior in the novel context. This experiment differed from Study 1 in that post-shock freezing behavior was permitted to fully develop out to 1 month post-injury before treatment. Sham operated rats (**Fig. 4**; dark and light grey bars) froze approximately 25% before treatment with MN166 or vehicle, while LFPI rats (**Fig. 4**; black and white bars) froze at significantly higher rates (~60%) in the novel context. Following treatment, LFPI+MN166 rat's freezing behavior was reduced to (~25%) compared to LFPI+vehicle rats (~50%). This effect was significant at 3 months, and remained so through 6 months following injury. Freezing in Sham+MN166 and Sham+vehicle rats could not be distinguished from LFPI+MN166 treated rats at all time-points following treatment, while LFPI+vehicle injected rats froze significantly more than both sham groups at all post-treatment time-points with the exception of LFPI+vehicle and Sham+vehicle, which did not differ at the 4 month and

Fig. 4

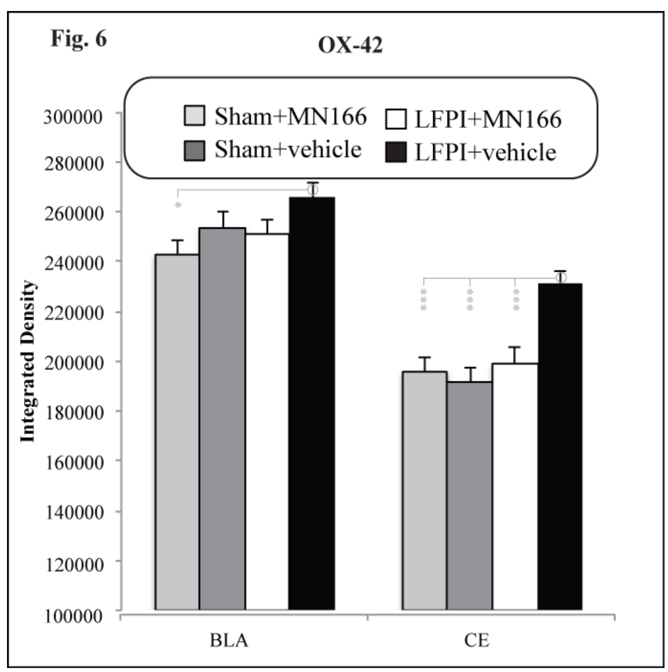


6 month time-points. Thus, MN166 injections after full development of post-traumatic anxiety had the effect of long-term reversal of anxiety symptoms, suggesting a reversible but otherwise prolonged and perhaps chronic neuro-inflammatory condition maintaining post-traumatic anxiety in untreated LFPI animals.

Reactive gliosis is reduced in amygdala and insula 6 months post-injury. Immunohistochemistry supports the possibility of chronic inflammation in untreated rats. LFPI+vehicle rats had significant increases in GFAP labeling in both insula and amygdala (**Fig. 5A & B,** respectively; black bars), indicating higher astroglial activation compared to sham operated and LFPI+MN166 treated rats. In the central amygdala (CE), microglial activation (**Fig. 6**; OX-42) was also greater in LFPI+vehicle injected rats compared to both sham operated groups and LFPI+MN166 treated rats, and was approaching significance for basolateral amygdala (BLA).



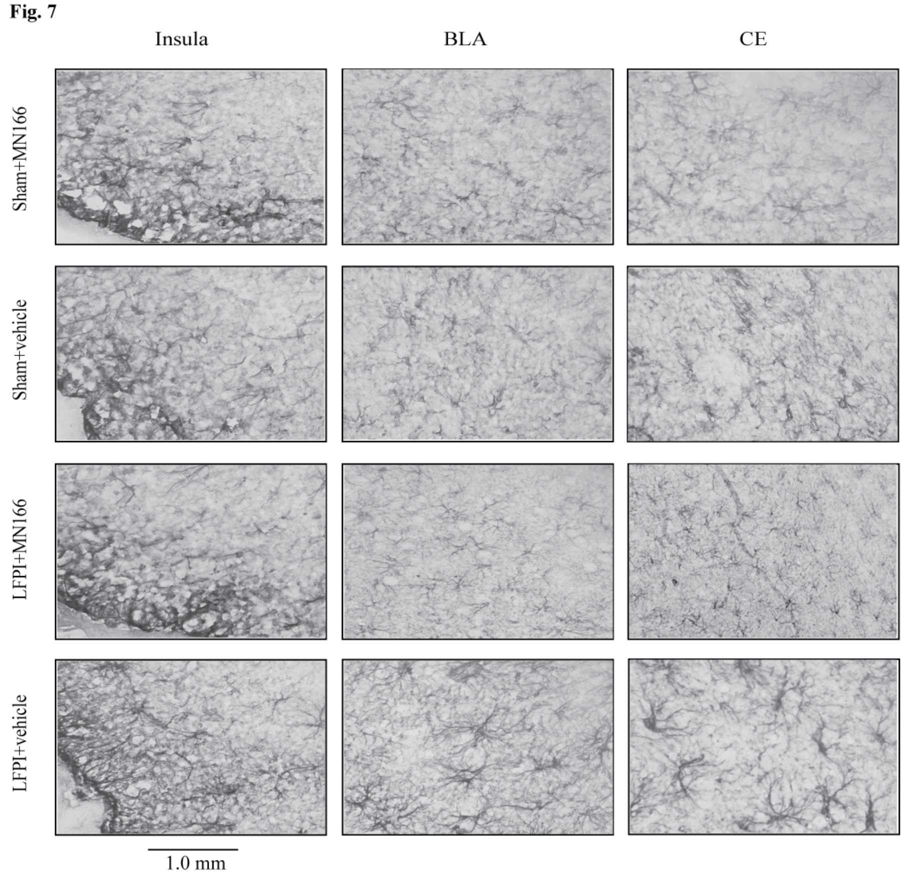
Astrocyte morphology 6 months post-injury. **Figure 7** shows representative images depicting GFAP immunoreactivity assessed in the amygdala and insula at 6 months post-injury. LFPI rats injected with vehicle showed clear signs of reactive astrocytes (bottom row), while sham operated rats appeared to have normal astrocyte morphology (top rows). LFPI rats treated with MN166 (third row) were difficult to differentiate from sham operated groups.



Relevence of our preliminary results to other work and the present proposal. Our first study demonstrates that LFPI induces anxiety-like behaviors in an animal model of post-traumatic anxiety. Further, we show the involvement of neuroinflammation by attenuating the behaviors and trauma-induced changes in reactive gliosis through MN166 based immunosuppression. Peri-injury administration results in a marked reduction in anxiety behaviors, which we suspect is due to attenuation of the inflammatory response post-injury, as astroglial and microglial activation is significantly reduced in the amygdala and insula, brain regions consistently implicated in anxiety in both rats and humans. This study is the first report of successfully reducing trauma-induced anxiety-like behavior based on immunosuppression. Lastly, this study validates the use of LFPI to induce anxiety-like behavior following experimental TBI in rats and introduces a model useful for exploring basic mechanisms of post-traumatic anxiety observed in humans.

Our second study replicates the finding of LFPI-induced increases in post-traumatic anxiety and shows that acute treatment with MN166 results in a reduction of post-traumatic anxiety and reactive gliosis in brain regions important to the development of anxiety. However, this study critically expands the clinical relevance of our previous findings because treatment was administered one month post-injury and the reductions in anxiety-like behavior persisted out to six months. The findings of elevated astroglial and microglial levels at time-points this long post-injury supports the hypothesis of chronic neuroinflammation in anxiety disorders following TBI. The results also broaden the critical treatment window for those with post-traumatic anxiety disorders and suggest neuroinflammation as a possible treatment target long after injury and behavior have been established. This is of potential clinical importance because studies indicate that prevalence rates continue to increase years after injury (87-89). Evidence for long-term risk in the development of post-traumatic anxiety documented in a study of TBI patients assessed 30 years following injury indicate that almost half (48.3%) of TBI participants develop a psychiatric disorder following injury, and almost half (23.3%) of the reported disorders are anxiety disorders, thus showing the importance of psychiatric follow-up and chronic treatment management after injury (89).

Fig. 7



Very few animal studies have reported success in treating post-traumatic anxiety. A recent meta-analysis (1989-2009, including 91 treatments and over 200 pre-clinical studies) assessed the impact of pharmacological agents on cognitive, motor and behavioral outcomes in rats following TBI, and the results revealed that almost no treatment improves behavioral outcomes, including anxiety, depression and aggression (90). Three studies that have been found to be effective in reducing anxiety-like aftermaths following TBI, as evidenced by increased exploratory behavior in open field and elevated plus tests in treated animals, utilized magnesium, resveratrol and progesterone (66, 79, 85). Although the mechanism of action for magnesium treatment is unknown, the neuroprotective effect is likely due to reductions in glutamate excitotoxicity, mitochondrial damage, and apoptosis (91). Resveratrol is a potent polyphenol with many antioxidant properties, which have been shown to decrease oxidative stress following TBI. Resveratrol has also been found to be neuroprotective against excitotoxicity, ischemia, and hypoxia (66). Progesterone is thought to protect against glutamate excitotoxicity by interacting with inhibitory $GABA_A$ receptors and through modulation of excitatory neurotransmitter (kainite, glycine, serotonin, and acetylcholine) receptors (91).

However, while the above treatments show reductions in anxiety-like behavior following TBI, all treatments require rapid administration (within 30 min – 6 h post-injury) reducing the therapeutic window to the day of injury. Additionally, pre-clinical studies utilizing animals have consistently failed to translate a successful pharmacological intervention to date, likely because highly controlled investigations may not be reflective of clinical trial designs. Pre-clinical studies tend to only include one injury severity (91). Another weakness in pre-clinical trials in animals is pre-treatment or very early interventions (30 min – 6 hours), in spite of evidence that many molecular, biochemical and immunological changes occur for many months to years following injury, and that clinical intervention may not be possible at such early stages of TBI. To better understand the pathophysiology of post-traumatic anxiety, pre-clinical treatments need to target longer treatment windows with range of injury severities. Our proposed project will attempt to overcome many of these limitations by focusing on delayed MN166 treatment and targeting mild, moderate and severe injuries, to extend our preliminary findings of neuroprotective immunosuppression on functional and immunological outcomes following TBI.

Uniqueness. We have developed a novel animal model of post-traumatic anxiety, using LFPI induced freezing behavior as a direct measure of fear responses. With this model, we show the first empirical proof of the theoretical concept that neuroinflammation plays a key role in post-traumatic anxiety and that a powerful post-injury intervention for post-traumatic anxiety development can be effected through brief peri-injury MN166 treatment. Our results represent a paradigm shift, challenging present attempts to treat post-injury anxiety symptoms, and moving instead toward preventing the development of post-traumatic anxiety in the first place by acutely suppressing the extreme and potentially chronic inflammatory response that TBI produces. Finally, and perhaps most exciting from both a scientific, clinical and military standpoint, we have preliminary evidence that acute treatment with MN166 at 1 month post-injury (after post-traumatic anxiety symptoms have fully developed), instead of at the time of injury, has the pronounced and long-term effect of reversing post-traumatic anxiety, which becomes indistinguishable from control animals. This finding suggests that post-traumatic anxiety is due in part to an ongoing chronic neuroinflammatory state, which may be disrupted by brief suppression of glia activation. To our knowledge, neuroimmune modulation as a means of post-traumatic anxiety prevention/treatment at time-points this late post-injury have never been tried before now. These results from our animal model form the basis of the present proposal to explore, for the first time, mechanisms of neuroimmune brain inflammation in the prevention and treatment of post-traumatic anxiety

2) HYPOTHESES

Hypothesis 1) Post-traumatic anxiety *prevention* across injury severity. We hypothesize that attenuating glial cell activation with MN166 4 days following mild, moderate, or severe LFPI will result in corresponding attenuation of anxiety, suggesting a preventative strategy for post-traumatic anxiety. Our expectations are based on the demonstrated success of peri-injury treatment in this model using moderate LFPI. They are also based on our success in reversing post-traumatic anxiety when already established at 1 month post-injury. Since our preliminary work was based on moderate LFPI, we expect significant if not equal improvement in post-traumatic anxiety symptoms with severe LFPI and equal if not better results with mild LFPI. Success with proximal post-injury treatment would present a far more clinically relevant finding for preventing post-traumatic anxiety and associated neuro-trauma without the need for pre-treatment. To test this hypothesis, we will examine the effects of LFPI and acute post-injury MN166 treatment on freezing behavior tested in a novel environment at 2 weeks, 1, 2, 3 and 6 months post-trauma.

Hypothesis 2) Post-traumatic anxiety *treatment* across injury severity. We hypothesize that post-traumatic brain inflammation is an ongoing, chronic process contributing to prolonged anxiety-like behavior, and that glial attenuation with administration of MN166 1 month following mild or severe LFPI, after the development of both behavioral and functional symptoms, will reverse or at least attenuate these symptoms, suggesting a treatment strategy for established post-traumatic anxiety. We have already had success at reversing established post-traumatic anxiety with treatment beginning at 1 month post-injury, this was evidenced out to long-term time-points, 6 months post-injury (**Fig. 4**). To test this hypothesis in mild and severe injury severities, we will use the same animal model and behavioral/histological evaluations as Hypothesis 1, but will delay treatment with MN166 until 1 month post-injury, when post-traumatic anxiety has fully developed. To test this hypothesis, we will examine the effects of LFPI and acute post-injury MN166 treatment post-traumatic anxiety-like behavior at 1-month through 6-months post-trauma.

3) TECHNICAL OBJECTIVES

The hypotheses outlined above lead to 2 specific aims addressing the following questions:
AIM 1) Can acute neuroimmune suppression begun 4 days following TBI prevent or at least attenuate development of post-traumatic anxiety and associated neuroinflammation? If so, is the effect long term (out to 6 months) and is prevention differentially effective for mild, moderate and severe TBI?
AIM 2) Can acute neuroimmune suppression begun 1 month following TBI reverse or at least attenuate ongoing post-traumatic anxiety and possible chronic neuroinflammation? If so, is the effect long term (out to 6 months) and is treatment differentially effective for mild, moderate and severe TBI?

4) PROJECT MILESTONES

We expect by the end of this 3 year project period to have determined: 1) the role of trauma-induced neuroinflammation in the development and/or maintenance of post-traumatic anxiety, 2) whether brain structures typically associated with anxiety in humans and in animal models reveal reactive gliosis in response to mild, moderate and severe injury, and 3) whether reduction of glial activation with MN166 holds promise for prevention and/or reversal of post-traumatic anxiety related symptoms.

As detailed in our Statement of Work, we expect to reach the first milestone of **AIM 1** within the second year of work. **Milestone #1:** Demonstration that early treatment with MN166 prevents or attenuates post-traumatic anxiety in mild, moderate, and perhaps severe TBI. We base this estimate on time required to perform preliminary studies of LFPI impact pressures required to reliably produce mild and severe TBI associated with post-traumatic anxiety (months 1-7), subsequent data collection for the first experiment (months 8-13; see 6 month procedural timeline for AIM 1; **Fig. 8**), followed by histology (months 14-18), data analysis (months 19-20), and publication (months 21-22). We expect to reach the second milestone on **AIM 2** by the end of the 3rd project year. **Milestone #2:** Demonstration that delayed treatment with MN166 reverses or attenuates established post-traumatic anxiety in mild, moderate, and perhaps severe TBI. This estimate is based on the 6-month procedural timeline for AIM 2 (months 23-28; **Fig. 9**), and additional time required for histology, data analysis, and publication (months 29-36).

5) MILITARY SIGNIFICANCE

Traumatic brain injury (TBI) has been described as the signature injury of the wars in the Middle East, where improvised explosive devices, suicide bomb blasts, and other combat related head trauma have seen a marked increase. This precipitous rise is highly correlated with a substantial increase of war fighters suffering from chronic PTSD, of which post-traumatic anxiety is the dominant symptom. There is considerable overlap in symptoms and high rates of co-occurrence between PTSD and post-traumatic anxiety. Soldiers reporting TBI are at very high risk for long-term mental and physical health problems in general, and the high rates of TBI and post-traumatic anxiety pose a particularly significant concern for the long-term health of U.S. veterans. A greater understanding of their interaction is critical to the treatment of Active Duty, Reserve, National Guard, and Veteran soldiers affected by TBI with psychiatric morbidity.

Our proposed project is directly relevant to two main USAMRMC research areas of interest. The first concerns the Combat Casualty Care Research Program (CCCRP) in that we plan to investigate the effectiveness of an orally available pharmacological intervention to limit the immediate, short- and long-term impairments that follow traumatic brain injury. The drug we will investigate, Ibudilast (MN166), is intended to mitigate post-injury neural and immune cell over stimulation, brain inflammation, resultant cell loss and neurologic dysfunction (resulting in post-traumatic anxiety). We will specifically explore the effectiveness of proximal (4-day) post-injury administration of MN166 on preventing/attenuating post-traumatic anxiety, an application well suited to first responders. MN166 is currently in FDA approved clinical trials for human pain management, so its potential translation to military treatment of post-traumatic anxiety would be far more facilitated compared to other potential neuroimmune suppressant compounds.

Our project is also directly relevant to 2 objectives of the Military Operational Medicine Research Program (MOMRP) under the categories of 1) Injury Prevention and Reduction, and 2) Psychological Health and Resilience, in that we focus on reducing the negative impact of concussion/mild traumatic brain injury by elucidating underlying mechanisms of post-traumatic anxiety associated with PTSD (neuroinflammation) and focusing on potential implementation of evidence-based prevention (immediate post-injury administration of MN166 to prevent post-traumatic anxiety) and treatment techniques (reversal of post-traumatic anxiety symptoms with neuroimmune attenuation well after post-traumatic anxiety symptoms have developed).

In this project, we will use an animal model of head trauma and post-traumatic anxiety to explore both its neurobiology and a means of prevention and treatment using suppression of inevitable trauma-induced brain inflammation. If successful, we expect our results to make a major scientific advance in understanding how post-traumatic anxiety occurs. But more importantly to injured soldiers, the drug we will test for prevention can be administered orally (i.e. pills), has been used for years in Asia (primarily for asthma treatment) with no known side effects, and, as noted above, is currently approved by the FDA for clinical trials concerning use in human chronic pain. Therefore our success should lead to very rapid translation of this work to a practical and effective prevention/attenuation of post-traumatic anxiety in battlefield emergency medicine, and treatment of already developed post-traumatic anxiety in the large Veteran population. This work has an urgent immediacy in that, not only are conflict induced brain injuries and cases of post-traumatic anxiety on the rise, but there is an unrelenting increase of the burden on Veteran health delivery with no effective treatment in sight. Finally, we expect success of this work to lead to additional experiments examining the effect of immunosuppression on other very common TBI-induced cognitive and motor disorders, which follow closely behind post-traumatic anxiety as major blast-injury related disabilities resulting from combat.

6) PUBLIC PURPOSE (written in laymen's terms)

Head trauma that can result from battlefield, sporting or automobile accidents frequently produces long-term injury to the brain. Patients with these brain injuries report not only a decreased ability to think and move properly, but also often report a profound and long-term feeling of anxiety that has devastating consequences to their employment and quality of life. While such post-traumatic anxiety has long been recognized, very little is known about how it is caused by brain injury and even less is known about how to either prevent its development and/or treat its symptoms. We have recently developed an animal model of post-traumatic anxiety in rats and determined that trauma results in prolonged inflammation of the brain, similar in some ways to commonly experienced inflammation of the skin in response to damage from burns. This is important because brain inflammation is well known to cause parts of the brain to become over active. We have also discovered that if we administer drugs that briefly suppress inflammation at the time of injury, or after post-traumatic anxiety has developed, the effect is to decrease over-activity of the brain and similarly decrease anxiety-like behaviors in rats. In this project we use our animal model to better understand the areas of the brain producing post-traumatic anxiety and to establish ways of preventing its development or reversing the symptoms once they occur. We will do this by decreasing inflammation produced by brain trauma with a powerful anti-inflammatory drug called "Ibudilast" that has already proven safe for use in humans.

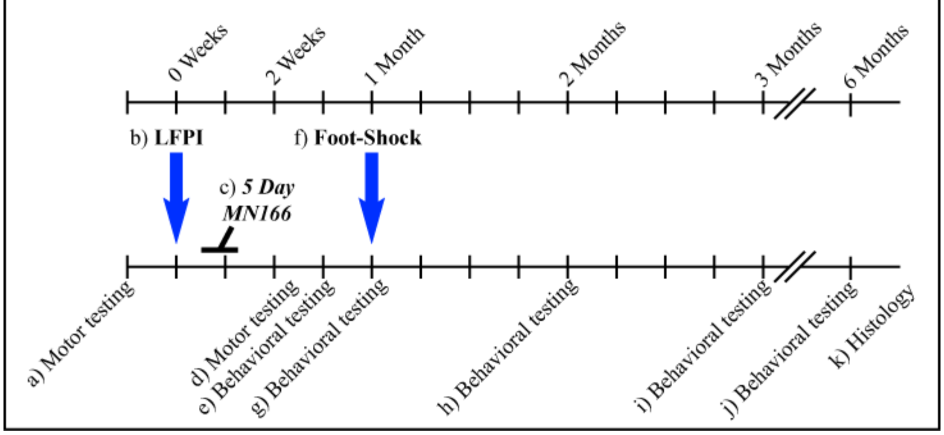
7) METHODS

<u>Subject groups and procedural timeline.</u>

(Details of methods indicated here with an "", are provided in "General Methods" below.)*

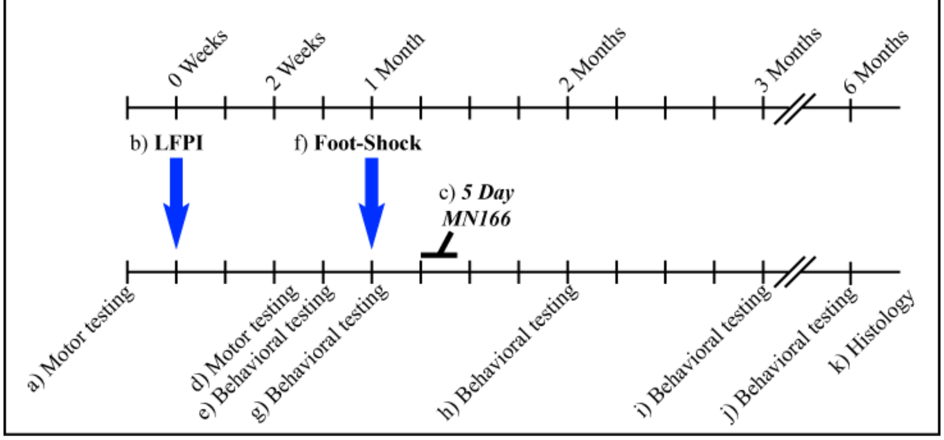
AIM 1) Post-traumatic anxiety prevention. All procedures will be performed in accordance with University of Colorado Institutional Animal Care and Use Committee guidelines for the humane use of laboratory rats in biological research. For the first hypothesis of this project, 80 adult viral-free male Sprague-Dawley rats (275-325g; Harlan Laboratories, Madison, WI) will be randomly assigned to 1 of 8 groups (10 rats each: Sham-operated/vehicle-injected, Sham-operated*/MN166-injected*, mildTBI*/vehicle-injected*, mildTBI/MN166-injected, moderateTBI*/vehicle-injected, moderateTBI/MN166-injected, severeTBI*/vehicle-injected, severeTBI/MN166-injected). Prior to TBI, baseline motor coordination testing* will be performed (**Fig. 8a**). TBI will then be induced as in our preliminary studies with LFPI* (**Fig. 8b**). At 4 days post-injury, daily subcutaneous injections of MN166* (in corn oil; 10 mg/kg) or vehicle will commence and be administered for 5 consecutive days (**Fig. 8c**). Motor testing* will be repeated following recovery and injections, beginning at 1-week post-injection (**Fig. 8d**; to eliminate the possible confound of motor impairment due to pain sensitivity from injections). The rats will be tested for % time freezing in a novel context* (minor stressor), exploration of an open field* and elevated plus maze* at 2 weeks post-injury (**Fig. 8e**) to establish baseline behaviors before foot shock* (i.e. anxiety-like behavior induced by LFPI alone without an added major stressor). As noted earlier, in our preliminary studies, examining a smaller number of rats, open field and elevated plus maze exploration showed results that trended in the same direction as freezing in the novel environment but did not reach significance. These additional measures are included in the present studies and are expected to reach significance with the larger number of rats per group. After baseline behavioral measurements, all animals will be exposed to foot shock* (**Fig. 8f**) in another context (to ensure the absence of contextual conditioning to the novel context) before retesting for the same anxiety-like behaviors at 1, 2 and 3 and 6 months post-injury (**Fig. 8g-j**). Tissue will then be collected* for histology (**Fig. 8k**).

Fig. 8



AIM 2) Post-traumatic anxiety reversal. To test the second hypothesis of this project, 80 adult viral-free male Sprague-Dawley rats (275-325g; Harlan Laboratories, Madison, WI) will be randomly assigned to 1 of 8 groups (10 rats each: Sham-operated/vehicle-injected, Sham-operated*/MN166-injected*, mildTBI*/vehicle-injected*, mildTBI/MN166-injected, moderateTBI*/vehicle-injected, moderateTBI/MN166-injected, severeTBI*/vehicle-injected, severeTBI/MN166-injected). Pre-injury motor testing (**Fig. 9a**), LFPI (**Fig. 9b**), post-injury motor testing (**Fig. 9d**), as well as the time-line for foot-shock (**Fig. 9f**) post-shock behavioral testing time-points (**Fig. 9h-j**) will be exactly the same as the time-line for AIM 1. However, a key difference in this experiment is that MN166 or vehicle injections will be delayed until behavioral symptoms of post-traumatic anxiety have been fully developed and tested at the 1 month post-injury time-point (**Fig. 9c**) to examine possible treatment related reversal or attenuation of anxiety-like behavior.

Fig. 9



***General Methods.**

Lateral Fluid Percussion Injury (LFPI). Rats are anesthetized with halothane (4% induction, 2.0-2.5% maintenance) and mounted in a stereotaxic frame. The LFPI adapted for our preliminary studies and the present proposal is widely used to induce TBI in animal models and has been described previously (62, 74, 92) utilizing a PV820 Pneumatic PicoPump (World Precision Instruments, Inc., Sarasota, FL) to deliver standardized pressure pulses of air to a standing column of fluid. A 3.0 mm diameter craniotomy is performed, with the exposed dura remaining intact. A female Luer-Loc hub (inside diameter of 3.5 mm) is secured over the craniotomy with cyanoacrylate adhesive. Following hub implantation, the animal is removed from the stereotaxic frame and connected to the LFPI apparatus. The LFPI apparatus will then be used to deliver a mild impact force (1.0 atmospheres; 10 ms), moderate impact force (2.0 atmospheres; 10 ms), or severe impact force (3.0 atmospheres; 10 ms). The injury cap is then removed, scalp sutured and the rats returned to their home cages for recovery. Sham operated rats undergo identical surgical preparation, but do not receive the brain injury.

Ibudilast (MN166) administration. MN166 (MediciNova, San Diego, CA) is a relatively non-selective phosphodiesterase inhibitor with anti-inflammatory actions via glial cell attenuation (93, 94). Treated rats receive a 5-day dosing regimen of once-daily MN166 (10 mg/kg, 1 ml/kg subcutaneously in corn oil) or vehicle (1 ml/kg subcutaneously, corn oil) injections beginning at 4 days (AIM 1) or 1 month (AIM 2) following LFPI. Weight will be recorded prior to each dosing and treatment administered at the same time each day to maintain constant levels across a 24 hr. period. Dose selection is based on prior animal pharmacology results (95), and has been shown to be safe and well tolerated, yielding plasma concentration-time profiles commensurate with high dose regimens in clinical development (96). MN166 administered via this regimen yields plasma and CNS concentrations that are linked to molecular target actions including, most potently, macrophage migration inhibitory factor (MIF) inhibition (97) and, secondarily, PDE's -4 and -10 inhibition (98). The relevance of MIF inhibition in disorders of neuroimmune function such as neuropathic pain has recently been well demonstrated (99). Such dosing regimens have clearly been linked to glial attenuation in other animal models (100). Our preliminary studies demonstrate that a dosing regime of 5 days at 10 mg/kg is well tolerated and yields significant effects.

Neuromotor Tests. Baseline testing of motor, vestibular and locomotive performance in all groups will be conducted immediately prior to surgery and again, at 1 week (Hypothesis 1) or 3 weeks (Hypothesis 2) following injury. These tests include ipsilateral and contralateral assessment of forelimb and hindlimb use to assess motor function, locomotion, limb use and limb preference (101, 102), toe spread to assess gross motor response (103), placing to assess visual and vestibular function (104, 105), catalepsy rod test to assess postural support and mobility (106), bracing to assess postural stability and catalepsy (107, 108) and air righting to assess dynamic vestibular function (109, 110). Scoring will range from 0 (severely impaired) to 5 (normal strength and function). The individual test scores will be summed and a composite neuromotor score (0–45) generated for each animal. In addition to the composite neuromotor score, limb-use asymmetry will be assessed during spontaneous exploration in the cylinder task, a common measure of motor forelimb function following central nervous system injury in rats (104, 111) and post-injury locomotor activity will be assessed through distance traveled on a running wheel, both tasks will be scored for 5 minutes under red light (~90 lux).

Freezing Behavior. A novel environment will be used to assess freezing behavior in response to a minor stressor (112). We and others have found that even though the environment cannot be considered novel after the first exposure, repeat testing at widely spaced (2-4 wk) intervals does not result in habituation of the freezing response (see **Fig. 1**) The environment will consist of a standard rat cage with one vertically and one horizontally striped wall. No aversive stimuli are introduced in this context and no conditioning should occur. Rats will be tested (5 minutes) and the percent of freezing behavior assessed. Freezing is defined as the absence of movement except for heart beat/respiration, and is recorded in 10 sec intervals.

Freezing behavior in the novel environment will be measured again following the administration of a foot shock in a separate shock apparatus. The shock apparatus consists of two chambers placed inside sound-attenuating chests. The floor of each chamber has 18 stainless steel rods (4 mm diameter), spaced 1.5 cm center-to-center and wired to a shock generator and scrambler (Colbourn Instruments, Allentown, PA). An automated program delivers a 2-sec/1.5 mA electric shock. Rats will be transported in black buckets and shocked immediately upon entry to chambers. Following shock, rats will be returned to their home cages.

Open field exploration. Spontaneous locomotor activities and exploratory behaviors will be evaluated using an open field testing apparatus (94). Rats will be placed facing the same direction, in the upper left corner of an open field-testing apparatus, consisting in a 40x40x40 cm Plexiglas enclosure. Rat displacements will be recorded for 10 min with a digital camera placed directly over the apparatus. The testing will begin as soon as the animal is placed in the open field. The camera will be attached to a computer running "Any-Maze Video Tracking SystemTM" (Stoelting Co., Wood Dale, IL, USA) program which tracks the animal and records its trajectory in the enclosure separated in 16 equal squares. The following parameters will be analyzed: entries into the inner arena, total distance traveled during the test period, vertical & horizontal locomotion, and freezing behavior.

Elevated plus maze exploration. The elevated plus maze is made of ivory Plexiglas with 50 cm long and 10 cm wide arms, elevated 50 cm above the floor. The closed arms are surrounded by a 50 cm wall. Rat displacements will be recorded for 10 min with a digital camera placed directly over the apparatus. The testing will begin as soon as the rat is placed in the central platform of the maze facing an open arm. The camera will be attached to a computer running "Any-Maze Video Tracking SystemTM" (Stoelting Co., Wood Dale, IL, USA) program which tracks the animal and records its trajectory in the enclosure. The following parameters will be analyzed: number of open arm entries, time spent in open/closed arms, distance traveled in open/closed arms, and freezing behavior.

Immunohistochemistry. Rats will be intracardially perfused with 0.9% saline and tissue collected, then fixed with 4% paraformaldehyde overnight at 4°C. Brain sections (20 μm) will be post-fixed with 4% PFA for 15 min at room temperature, then treated with 0.03% H_2O_2 for 30 min. Immunoreactivity in brain regions associated with anxiety (insula and amygdala) will be assessed for markers of microglia (CD11b/c; OX42 labeling) and astrocytes (glial fibrillary acidic protein; GFAP), using an avidin-biotin-horseradish peroxidase (ABC) reaction (113). Briefly, the sections are incubated at 4 °C overnight in either mouse anti-rat OX-42 (1:100; BD Biosciences Pharmingen, San Jose, CA) or mouse anti-pig GFAP (1:100; MP Biomedicals, Aurora, OH). The following day, sections are incubated for 2 h with biotinylated goat anti-mouse IgG antibody (1:200; Jackson ImmunoResearch, West Grove, PA). Sections are then washed and incubated for 2 h at room temperature in ABC (1:400 Vector Laboratories, Burlingame, CA) and reacted with 3', 3-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO). Sections are air-dried over night, dehydrated with graded alcohols, cleared in Histoclear, and cover slipped with Permount (Fisher Scientific, Fairlawn, NJ). Densitometry analysis is performed using Scion Image software.

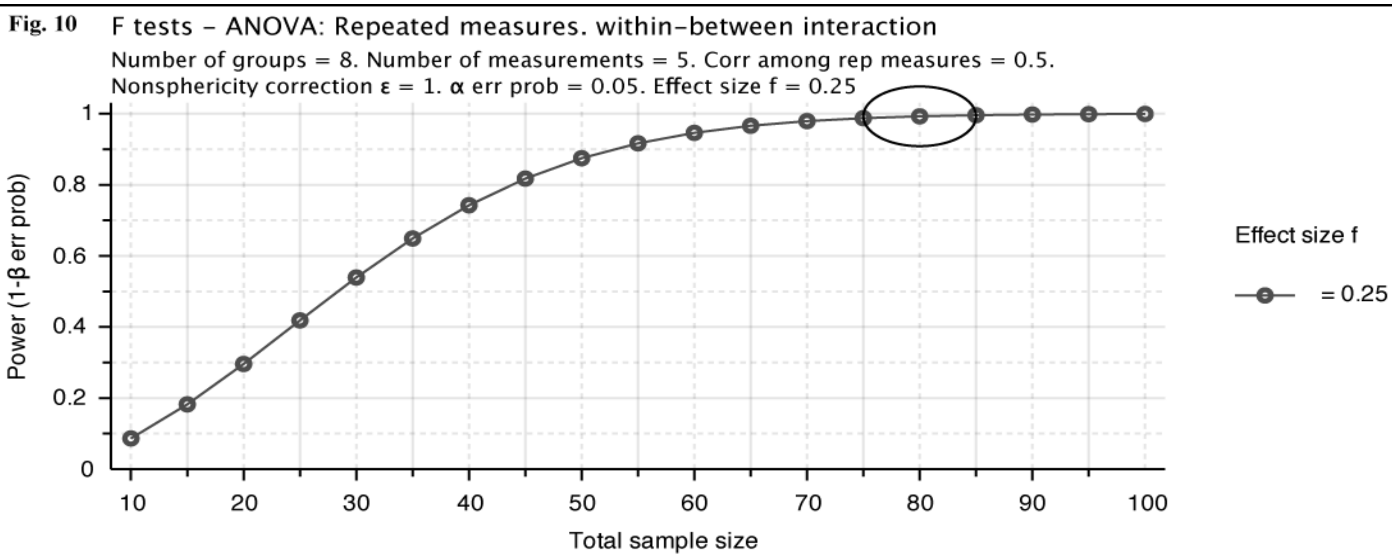
Analytic plan.

Image Analysis. Slides will be viewed with a Nikon Ci-LE System with Motorized nosepiece, X, Y, and Z system and camera with bright-field illumination at 10X magnification. Densitometry analysis is performed using Scion Image software. Images are opened in ImageJ, converted into gray scale and rescaled from inches to pixels. Background areas are chosen in the white matter or in cell-poor areas close to the region of interest (ROI). The number of pixels and the average pixel values above the set background are then computed and multiplied, giving an integrated densitometric measure. Six measurements are made for each ROI; the measurements are then averaged to obtain a single integrated density value per rat, per region.

Statistical analyses. Analyses of behavioral measures will use repeated measures (time point post-injury) ANOVAs, with group assignment as the independent variable, followed by Bonferroni post hoc tests for multiple comparisons of individual time points. Immunohistochemistry data will only be collected at one time point (6 months post-injury) and one-way ANOVAs will be conducted, with group assignment as the independent variable. Differences with a p-value of <0.05 will be considered significant.

Power Analysis. Power analysis of our 8 x 5 design (group: 8 levels, and time: 5 levels), for AIM 1 & 2 (**Fig.**

Fig. 10



10) indicates that acceptable power of 0.8 can be achieved with 45 rats and the maximum power of 1.0 will be reached with N = 80 rats as planned (**Fig. 10**; oval).

Expected challenges.

Based on our preliminary data, we expect that moderate-severe LFPI in both AIM 1 & 2 will reliably result in increased post-traumatic anxiety behavioral symptoms in untreated LFPI rats. However, a potential challenge could be that mild TBI at the 1.0 atmosphere impact pressure does not result in anxiety-like behavior. A solution to this problem would be to increase the impact pressure to 1.5 atmospheres, which is on the higher end of mild TBI in rodent models. However, recent studies have reported anxiety-like behaviors following mild TBI (86) and collaborative work between our laboratory and Dr. Watkins's laboratory has revealed anxiety-like behavior at the 1.0 atmosphere impact pressures suggested here.

Another potential challenge could be unacceptable variability of our LFPI results due variability of the injury. This has been minimized by the use of calibrated and temporally precise pressure pulses delivered by a Pneumatic PicoPump. However, in the course of our preliminary studies, we have noted 2 sources of injury variability that we will control in the present studies. The first is direct damage to the middle cerebral artery (MCA) that results in a large sub-dural hematoma and loss of adequate blood supply to large regions of cortex. The second is rupturing of the dura, simulating a (more severe) penetrating head wound instead of the closed head injury intended by the LFPI model. To avoid MCA damage, we will use rats of 275-325 gm., which, in combination with our lateral craniotomy location, minimize this outcome. In occasional cases where the MCA can be visualized within the area of craniotomy, the rat will be immediately sacrificed and replaced. Dural rupture is apparent following LFPI as extradural bleeding. These animals will also be replaced at the time of surgery.

Motor impairments have been found following severe LFPI, and impairments such as catalepsy, could confound our behavioral measures. Early pilot studies in our laboratory revealed motor impairments following surgery; however, changing the site of injury to a more lateral location and avoiding disruption of the MCA, eliminated these deficits. We will include a series of motor coordination tests to ensure that subsequent results aren't driven by motor impairments. In addition to motor impairments, these tests will assess deficits in other systems that may affect behavior, including vestibular, visual and proprioceptive systems. Animals found to have significant neuromotor composite scores compared to baseline scores will be excluded from the study.

Finally, animals have an increased risk for infection as a result of the surgery, which would increase inflammation and potentially cause problems with our behavioral measures. Therefore, our staff and campus veterinarian will carefully monitor the animals for increases in weight loss and temperature, the two most common indicators of the presence of infection. Animals will be excluded from the study if their weight drops below 20% of their baseline weight and/or temperature increases above 39°C, guidelines given to us by our on-staff veterinarian. We practice sterile surgical techniques to minimize sources of infection, and have not had to exclude any animals due to infections in our preliminary studies.

Expected outcomes and consequences of success/failure.

AIM 1) Post-traumatic anxiety prevention.

Benchmark for success-> We will conclude that suppressing glial activation with acute MN166 administration is an effective means of preventing development of post-traumatic anxiety if anxiety behavior in LFPI-MN166 treated rats at the 6 month post-injury time-point is significantly less than LFPI-untreated rats and cannot be distinguished from sham-operated controls in at least one of the TBI severity levels.

Consequence of this success-> This outcome alone would carry promise for post-traumatic anxiety prevention and would inspire more detailed future investigation of dose–response curves to determine an optimum treatment regime (duration and dose) and critical treatment windows (how soon after TBI) for maximum preventative effect.

Benchmark for success-> It is possible that treatment following TBI by 4 days as planned will not be as effective in attenuating post-traumatic anxiety symptoms as that seen in our preliminary study which began

injections 1 day prior to injury. Yet, if the result of treatment in LFPI rats is post-traumatic anxiety behavior that is significantly less than untreated rats (however, significantly more than sham-operated controls), we will still consider this outcome to be a success in that any significant improvement would be of potential translational significance.
Consequence of this success-> The ability to significantly attenuate the development of post-traumatic anxiety in our animal model will motivate future investigation of dose–response curves as noted above to see if treatment effects can be further increased and perhaps lead to actual prevention. Depending on the success of AIM 2, it would also be critical to further evaluate combining partial prevention with subsequent treatment (i.e. with additional delayed inflammatory attenuation) to see if the combination is more effective than either approach alone. It should be emphasized that since protracted MN166 treatment is well tolerated by humans and the compound can be effectively administered orally, long duration treatment beginning soon after TBI and continuing for months post-injury could be a distinct translational possibility.
Consequence of failure-> It is possible that MN166 treatment at 4 days following impact will have no influence on post-traumatic anxiety at any of the TBI severity levels. While this would call our hypothesis into question, it would have no impact on the justification for our AIM 2 study since the mechanisms and efficacy of prevention may differ from those of reversal. However, if a failure in preventing behavioral symptoms was also correlated with a failure to decrease gliosis in histological analysis, it would suggest that our hypothesis may be valid but our treatment was not, indicating future examination of other glial modulating drugs (for example, SLC022; "propentofylline"; 3-methyl-1-(5-oxohexyl)-7-propyl-3,7-dihydro-1H-purine-2,6-dione) that, while not as close to human translation as MN166, could further our understanding of potential neuroimmune prevention of post-traumatic anxiety.

AIM 2) Post-traumatic anxiety treatment/reversal.
Benchmark for success-> We will conclude that attenuation of glial activation with acute MN166 administration, after behavioral symptoms of post-traumatic anxiety have developed, is an effective means of reversing post-traumatic anxiety if anxiety behavior in LFPI-treated rats at the 6 month time-point is significantly less than LFPI-untreated rats and cannot be distinguished from sham-operated controls in at least one of the TBI severity levels.
Consequence of this success-> As noted, this outcome is independent of the success or failure of AIM 1 since the mechanisms and timing of prevention versus reversal may differ. Successful reversal would directly motivate follow-up studies looking at much longer duration post-injury delays before treatment (we have recorded large and persistent freezing responses in our rat post-traumatic anxiety model up to 18 months post-injury) to see if potential reversal might be eventually applicable to patients with ongoing and chronic post-traumatic anxiety.
Benchmark for success-> Similar to our AIM 1 benchmark, it is possible that delayed treatment will significantly attenuate, but not reverse, post-traumatic anxiety behavior in this study. This outcome would not be surprising since, particularly at later time points post-injury, it is likely that ongoing brain inflammation is only one of several contributors to behavioral symptoms. There is a large body of evidence showing that increased oxidative and nitrosative stress comprise a secondary injury cascade following TBI. Permanent damage to brain structures following TBI is likely, due to increases in the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are initiated by inflammatory mediators following activation of immune system in response to insult (26, 42, 43) and result in oxidative damage that can disrupt neuroprotective functioning. We may succeed in interrupting further damage but not reversing that which has occurred, leaving substantial residual deficits and post-traumatic anxiety behavior.
Consequence of this success-> A partial reversal of post-traumatic anxiety would still be of great importance both for understanding the role of ongoing neuroimmune contributions and for possible translational studies concerned with treatment. As noted earlier, depending on the outcome of AIM 1, this result would also motivate examination of the effectiveness of combined treatment both soon after injury and at later post-injury time-points when behavioral symptoms of post-traumatic anxiety may yet emerge.
Consequence of failure-> Failure to find any influence of glial attenuation on reversing established post-traumatic anxiety behavior in any of the TBI severity levels would support our null hypothesis, that the

contributions of brain inflammation are no longer contributing to chronic post-traumatic anxiety at later time-points. However, as noted for AIM 1, if a failure to attenuate post-traumatic anxiety symptoms is also associated with failure to decrease gliosis, we would conclude that our treatment was ineffective and explore other glial modulating compounds that may be have more potent anti-inflammatory effects.

Investigator-Initiated Research pre-proposal to the Peer Reviewed Medical Research Program

Topic Area: This proposal directly addresses one FY13 PRMRP Areas of Interest, epilepsy.

Research Idea: Approximately 50 million people worldwide have epilepsy [1] with 100,000 new cases diagnosed annually in the United States alone [2]. Traumatic brain injury (TBI) has been described as the signature injury of modern conflicts such as those in Afghanistan and Iraq[3]. TBI is also a major cause of acquired epilepsy, with overt seizures reported in up to 50% of survivors [4] and with approximately 20% of all symptomatic epilepsies attributed to TBI [5]. In addition, unlike many other forms of symptomatic epilepsies, fully developed post-traumatic epilepsy (PTE) is particularly difficult to treat both medically and surgically[6]. One way acquired epilepsies such as PTE and temporal lobe epilepsy (TLE) are distinguished from idiopathic epilepsies is that they are typically precipitated by an identifiable brain insult (i.e. traumatic brain injury or status epilepticus), followed by a prolonged "latent" period of <u>epileptogenesis</u> before the appearance of the first spontaneous behavioral seizures. The epileptogenic period provides a unique opportunity for discovery of anti-epileptogenic strategies, however progress in this area of clinical research has been hindered by the lack of reliable biological identifiers that can be used to measure and quantify the progress of epilepsy or its treatment (<u>biomarkers</u>) during this electrographically "silent" period.

Understandably, traditional emphasis has been placed on development of anti-seizure treatments for intervention once recurrent spontaneous seizures have been clinically established[7], however, the window of intervention, prevention, or mitigation may occur during the clinically silent period of epileptogenesis (often many months to years post-insult). A change in focus from symptom mitigation, to disease prevention, may therefore be advantageous, but in order for this to happen, novel and reliable biomarkers must be developed in order to allow investigation of this clinically silent period[8]. To date, however, most research conducted during the latent period, has focused on passive brain measures such as EEG, MRI, epileptiform spike and serum analysis, or invasive measures, such as gene expression and cellular markers to assess changes in brain physiology. We postulate that failure in the development of new compounds for the prevention, and often the treatment, of epilepsy may be due, in part, to a lack of brain markers that allow scientists and clinicians to <u>actively probe</u> the central nervous system during epileptogenesis and thereby quantify disease progression and treatment efficacy. The establishment of active biomarkers that examine changes occurring during the epileptogenic period (and beyond) would be beneficial in two key areas; **1)** investigation of the underlying mechanisms of epileptogenesis leading to more effective treatment regimes targeting prevention **2)** use as diagnostic tools to predict the risk/probability of acquiring epilepsy in high risk patients following brain insults. Indeed, the later could prove immediately beneficial for warfighters and veterans due to the high occurrence of head trauma in modern warfare [4], the long epileptogenic period observed in these cases (months to years), and the high cost of treatment of epilepsy once it is established estimated to cost the US government up to 15.5 billion a year [2]. In this project we will concentrate on identification of novel biomarkers, with immediate human translational feasibility that can be used as, low-cost, noninvasive, biological assays that reflect alterations in brain functionality, induced by brain insult. The long-term goal of our research plan is to discover epileptogenic biomarkers in rodent models of epilepsy that can help further uncover the inherent mechanisms underlying epilepsy, used to monitor and <u>quantify</u> the efficacy of novel antiepileptogenic therapies during the electrographically/clinically silent period, and which can quickly be translated to human clinical use to screen high risk military service members, veterans and beneficiaries who have received a brain insult to asses if they are at risk for developing epilepsy.

Research Strategy:

To this end our lab has recently uncovered a novel biomarker of epileptogenesis in the lithium-pilocarpine (Li-pilo) model of epilepsy in rats. Using chronically implanted electrodes we have measured the auditory evoked response, from a click stimulus, in both primary auditory cortex as well as in the hippocampus. The averaged auditory evoked potential from 128 clicks was collected every 30 minutes from rats under 24/7 video and electrophysiological monitoring. The auditory stimulus has a few distinct advantages as a probe of neural circuits, 1) it is non-invasive, 2) it can be presented chronically without disruption of the animals normal sleep/wake activity if the volume is at low enough levels[9], 3) it can be presented to freely moving rats, 4) due to its non-invasive nature it has translational capability. Under baseline conditions there is a highly stereotyped auditory evoked potential (AEP) in both the primary auditory cortex as well as the hippocampus. The archetypal AEP morphology in sensory responsive brain regions, reflecting normal underlying neuronal circuit function, offers a stable criterion against which later changes in brain function can be measured. By monitoring rat AEP's 24/7 in the Li-pilo model of epilepsy we have discovered that the normal archetypal morphology of these sensory evoked brain events undergoes systematic and stereotyped changes during the latent period, which predict the probability of the development of later spontaneous seizures. Chronic and progressive changes in AEP waveform during epileptogenesis in animals that eventually develop spontaneous recurrent seizure (SRS) are distinguished from the AEP in animals that never develop epilepsy in that the latter return to a near baseline state. Interestingly, in animals that develop epilepsy, the changes in morphology of the AEP (repeatably progressive changes in the latency, duration and amplitude of temporal components) <u>continue long past the first SRS</u>, suggesting an ongoing epileptogenic period beyond the typically defined latent period. This prolonged epileptogenic period, sensitively indexed by continued alteration of the AEP, is recently suggested in the literature[10] based on seizure progression alone, and indicates not only the possibility of progressive epileptogenesis that continues beyond the first SRS, but also the potential for antiepileptogenic treatment even following the onset of SRS with a means of monitoring success. The objective of this proposal is to further explore and characterize this novel biomarker of epileptogenesis with 3 experiments.

Specific Aim 1: Fully characterize the morphological alternations that occur in cortical and hippocampal auditory evoked responses following brain insult from lithium-pilocarpine.

Specific Aim 2: Further probe the glutamatergic cell population responsible for the AEP in isolation from the auditory sensory pathway using optogenetically-evoked responses directly in the hippocampus and cortex.

Specific Aim 3: Probe for archetypal morphological alterations in both auditory and optogenetically evoked responses in hippocampus and cortex following brain insult from lateral fluid percussion injury.

Experimental approach: Aim 1) Male Sprague Dawley rats (250-300g) will be randomly assigned to three groups, 1) Li-pilo, 2) Li-saline, and 3) no-injection. All animals will then be implanted with one SS screw electrode over primary auditory cortex and one bipolar electrode in dorsal hippocampus. After a 3-week recovery from surgery, animals will be attached to a head mount for chronic tethered video/EEG recording. Along with continuous EEG, averaged auditory evoked potentials (AEP) will be recorded every 30 minutes. Following a 2 week baseline animals will receive either the Li-pilo protocol[11], the Li-pilo protocol with saline in the place of pilocarpine, or no injections. Continuous recordings will be performed for 3 months. Animals will then be sacrificed and mossy fiber sprouting (classic characteristic of epileptic hippocampus) will be assessed with Timm staining. EEG will be visually scanned for seizures and severity classified based on associated video records. Both spontaneous EEG and AEPs will be compared to baseline recording throughout epileptogenesis and beyond. EEG will be assessed for band-delimited changes in power spectral density, as well as epileptiform spike density (based on automated template matching software written by the P.I. and currently in use). Changes in AEP waveform morphology will be quantified according to component peak amplitudes, duration, and post-stimulus latency. An additional cohort of li-pilo animals will have microelectrodes chronically implanted in the hippocampus and cortex to characterize the multiunit activity (to assess changes in excitatory/inhibitory balance) associated with the components of the AEP during baseline, epileptogenesis and SRS. We expect it will require 12 months to complete Aim 1.

Aim 2) The li-pilo model will be used again, but optogenetically evoked local field potentials (oLFP) will be added. Three groups, similar to those in aim 1, will be used with half of each group receiving excitatory opsin ChR2 (AAV-CaMKIIa-hChR2(H134R)-EYFP) injections into the target structures (cortex and hippocampus) at the time of the surgical implantation of the electrodes, and the other half receiving a control AAV-fluorophore (AAV-CaMKIIa-EYFP). These optogenes will be targeted at the glutamatergic neuronal population using the CaMKIIa promoter. Multimode fiber optic fibers will be glued to the electrodes to allow photo-stimulation (473nM, 20-35mW/mm^2) of the target structures. Photo-stimulation to generate an averaged oLFP will occur every 30 minutes following auditory stimulation, for the duration of the study (3 months). Care will be taken to stimulate at frequencies known to not influence synaptic plasticity, however recent optogenetic kindling studies offer the possibility that after-discharge from high frequency stimulation could be used as an additional measure of excitability in a separate cohort[12]. Baseline and li-pilo time course and analysis will be identical to aim 1. Analysis of changes in oLFP waveform morphology will be quantified according to component peak amplitudes, duration, post-stimulus latency, and will be compared to concurrent changes in AEP morphology. An additional cohort of li-pilo/AAV-CaMKIIa-hChR2(H134R)-EYFP animals will have microelectrodes chronically implanted in the hippocampus and cortex to characterize the multiunit activity associated with the components of the oLFP during baseline, epileptogenesis and SRS. We expect it will require 12 months to complete Aim 2.

Aim 3) The same protocol as aim 2, but the li-pilo model will be substituted with the later fluid percussion traumatic brain injury model (LFP, a model with which we have 4 years experience[13]). The animals will be randomly assigned into 4 groups 1) LFP/AAV-ChR2, 2) shamLFP/AAV-ChR2, 3) LFP/AAV-EYFP, 4) shamLFP/AAV-EYFP. Following baseline recording of 2 weeks LFP will be delivered. Signs of epileptogenesis will be monitored using AEP and oLFP for 8 months. We expect it will require 12 months to complete Aim 3.

Impact: Civilian patients that receive severe TBI are 30 times more likely to develop epilepsy, and soldiers who have sustained blast injury are at even greater risk [4,6]. Because of the nature of modern conflict and the long duration of the latent period in humans (months to years), it can be expected that many veterans and active duty warfighters will develop epilepsy, in addition to those that already suffer from it [3,4]. This long latent period offers some advantages though, if AEP biomarkers can be further explored and characterized, it is very possible that they could be used to screen high-risk individuals who have been exposed to brain trauma[8]. By using a well characterized model of epilepsy (li-pilo), with a relatively short latent period and high rate of SRS, to initially characterize this novel biomarker then extending these findings to the slower model of TBI induced epilepsy (LFP), we hope to speed up the potential of this biomarker for translational use. Inexpensively measured AEP's in individuals exposed to brain trauma could lead to enhanced anti-epileptogenic strategies by identifying those individuals most vulnerable to epilepsy development before the manifestation of spontaneous seizures.

Submitted to J. Neurotrauma

Delayed, post-injury neuroimmune suppression reduces anxiety-like behavior following lateral fluid percussion injury in rats

Krista M. Rodgers, Ph.D.[1], Yuetiva K. Deming, B.A.[1], Florencia M. Bercum, B.A.[1], Serhiy Y. Chumachenko, B.A.[1], Julie L. Wieseler, Ph.D.[1], Jerry W. Rudy, Ph.D.[1], Kirk W. Johnson, Ph.D.[2], Linda R. Watkins, Ph.D.[1] and Daniel S. Barth, Ph.D.[1]

[1]Department of Psychology and Neuroscience, University of Colorado, Boulder, CO, U.S.A., [2]MediciNova, Inc., La Jolla, CA, U.S.A.

Authors

Krista M. Rodgers

University of Colorado, Department of Psychology and Neuroscience, UCB 345

Boulder, CO 80309, USA

Phone: 303-492-0359, Fax: 303-492-2967, Email: Krista.Rodgers@colorado.edu

Yuetiva K. Deming

University of Colorado, Department of Psychology and Neuroscience, UCB 345

Boulder, CO 80309, USA

Phone: 303-492-0359, Fax: 303-492-2967, Email: Yuetiva.Deming@colorado.edu

Florencia M. Bercum

University of Colorado, Department of Psychology and Neuroscience, UCB 345

Boulder, CO 80309, USA

Phone: 303-492-0359, Fax: 303-492-2967, Email: fbercum@gmail.com

Serhiy Y. Chumachenko

University of Colorado, Department of Psychology and Neuroscience, UCB 345

Boulder, CO 80309, USA

Phone: 303-492-0359, Fax: 303-492-2967, Email: Serga.Chumachenko@gmail.com

Julie L. Wieseler

University of Colorado, Department of Psychology and Neuroscience, UCB 345

Boulder, CO 80309, USA

Phone: 303-492-0359, Fax: 303-492-2967, Email: Julie.Wieseler@colorado.edu

Jerry W. Rudy

University of Colorado, Department of Psychology and Neuroscience, UCB 345

Boulder, CO 80309, USA

Phone: 303-492-3306, Fax: 303-492-2967, Email: Jrudy@colorado.edu

Kirk W. Johnson

MediciNova, Inc.

4350 La Jolla Village Drive, Suite 950

La Jolla, CA, 92122, USA

Phone: 858-373-1500, Fax: 858-373-7000, Email: kjohnson@medicinova.com

Linda R. Watkins

University of Colorado, Department of Neurology

Boulder, CO 80309, USA

Phone: 303-492-7034, Fax: 303-492-2967, Email: Linda.Watkins@Colorado.EDU

Daniel S. Barth, PhD. (Corresponding author)

University of Colorado, Department of Psychology and Neuroscience, UCB 345

Boulder, CO 80309, USA

Phone: 303-492-0359, Fax: 303-492-2967, Email: dbarth@psych.colorado.edu

Pages: 36

Figures: 4

Word count: abstract: 219, introduction: 496, discussion: 1454

Conflict of interests: Kirk W. Johnson is chief medical officer of MediciNova, Inc., the pharmaceutical firm providing MN166 for this research.

Acknowledgements: US Army Medical Research and Material Command grant PR100040, Craig Hospital Gift Fund, University of Colorado Innovative Seed Grant, Autism Speaks Pilot Study grant 7153, and National Institutes of Health grant NS36981 to DSB, and National Institutes of Health grants DA024044, DA01767 to LRW.

Abstract

Traumatic brain injury (TBI) increases the risk of neuropsychiatric disorders, particularly anxiety disorders. Yet, there are presently no interventions to prevent development of post-traumatic anxiety or effective treatments once it has developed. This is due in large part to a lack of understanding the underlying physiological mechanisms. Recent research suggests that neuroinflammatory responses to injury may play a role in at least the development of post-traumatic anxiety in animal models. Acute peri-injury administration of anti-inflammatory compounds such as Ibudilast (MN166), prevent reactive gliosis associated with innate and adaptive immune responses and also prevent anxiety-like freezing behavior that typically results from lateral fluid percussion injury (LFPI; an animal model of human traumatic brain injury) in the rat.

There is clear evidence in both human and animal studies that post-traumatic anxiety, once developed, is a chronic, persistent, and drug refractory condition. In the present study, we sought to determine if neuroinflammation could contribute to the long-term maintenance of post-traumatic anxiety. We examined the efficacy of acute (5-day) anti-inflammatory treatment in decreasing anxiety-like freezing behavior when introduced at one-month following injury. Delayed treatment substantially reduced established anxiety-like freezing behavior due to LFPI, and continued neuroprotective effects were evidenced six-months post-treatment. These results support the conclusion that neuroinflammation may be involved in both the development and maintenance of anxiety-like behaviors following TBI.

Introduction

Over 5.3 million people in the U.S. are living with traumatic brain injury (TBI)-related disabilities (Faul, 2010) including anxiety disorders, which are among the most prevalent (Rao and Lyketsos, 2000; Hiott and Labbate, 2002; Vaishnavi et al., 2009). The risk for developing post-traumatic anxiety remains elevated for years post-injury (Morton and Wehman, 1995; Deb et al., 1999; Koponen et al., 2002). As the temporal pattern of onset is variable and the etiology unclear, there are currently no interventions to prevent development or provide treatment for post-traumatic anxiety.

Neuroinflammation is emerging as a therapeutic target for preventing the development of post-traumatic anxiety. Damaged cells produce danger signals, which relay and amplify immune responses (Pugin, 2012). Glial cells undergo marked recruitment and activation in response to danger signals (Matzinger, 1998; Hirsiger et al., 2012) secreting proinflammatory cytokines and chemokines (Gehrmann et al., 1993; Gehrmann et al., 1995). These inflammatory agents destabilize neurotransmitter balance, negatively impact neuronal survival, and therefore may contribute to functional alterations of brain areas involved in post-traumatic anxiety (Sternberg, 1997; Raison and Miller, 2003; Szelenyi and Vizi, 2007).

Several studies have reported increased anxiety-like behavior in rodent TBI models (Connor et al., 1998; Cragnolini et al., 2006; Sokolova et al., 2007; Zubareva and Klimenko, 2009) including increased conditioned (Reger et al., 2012) and unconditioned (Rodgers et al., 2012) fear responses to both learned and novel stimuli. TBI in rodents also increases levels of activated glial cells and proinflammatory cytokines (Chen et al., 2007; Homsi et al., 2009; Li et al., 2009; Homsi et al., 2010; Rodgers et al., 2012), and administration of these cytokines increases anxiety-like behaviors (Connor et al., 1998; Cragnolini et al., 2006; Sokolova et al., 2007; Zubareva and Klimenko, 2009). We have recently demonstrated acute peri-injury administration of the glial activation inhibitor, Ibudilast (MN166), prevents reactive gliosis and development of anxiety-like freezing behavior in rats (Rodgers et al., 2012).

Glial activation continues for months to years following injury and may be involved in the development and maintenance of post-traumatic anxiety (Gentleman et al., 2004; Streit et al., 2004; Nagamoto-Combs et al., 2007; Ramlackhansingh et al., 2011). Mounting evidence supports the role of inflammatory processes in both TBI and anxiety disorders, but there is an absence of research focused on neuroinflammation in patients with comorbid TBI/anxiety because studies typically exclude patients with a history of TBI and vice versa due to difficulties in evaluation and overlapping symptoms. Yet, evidence for chronic inflammation has been found in a number of studies examining patients with post-traumatic stress disorder (PTSD), panic disorder (PD), and other anxiety disorders (Spivak et al., 1997; Rohleder et al., 2004; Tucker et al., 2004; Konuk et al., 2007; von Kanel et al., 2007; Hoge et al., 2009). The objective of the present study was therefore to determine if neuroinflammation could contribute to the long-term maintenance of post-traumatic anxiety in an animal model. We examined the efficacy of delayed, immunosuppressive treatment in reversing established anxiety-like behaviors and reducing TBI-induced immunological damage.

Materials and Methods

Twenty-four adult viral-free male Sprague-Dawley rats (275-325g; Harlan Laboratories, Madison, WI) were housed in pairs in temperature (23 ± 3 °C) and light

(12:12 light: dark) controlled rooms with *ad libitum* access to food and water. All procedures were performed in accordance with University of Colorado Institutional Animal Care and Use Committee guidelines for the humane use of laboratory rats in biological research. Rats were randomly assigned to the following groups (n = 6/group): sham operated+vehicle, sham operated+MN166, LFPI+vehicle and LFPI+MN166.

Lateral Fluid Percussion Injury

LFPI rats were anesthetized with halothane (3% induction, 2.0-2.5% maintenance) and mounted in a stereotaxic frame. The lateral fluid percussion injury used in this study has been described previously (McIntosh et al., 1989; Thompson et al., 2005; Frey et al., 2009). Briefly, a 3.0 mm diameter craniotomy was centered at 3 mm caudal to bregma and 4.0 mm lateral of the sagittal suture, with the exposed dura remaining intact. A female Luer-Loc hub (inside diameter of 3.5 mm) was secured over the craniotomy with cyanoacrylate adhesive. Following hub implantation, rats were removed from the stereotaxic frame and connected to the LFPI apparatus. The LFPI apparatus delivered a moderate impact force (2.0 atmospheres; 10 ms). The injury cap was then removed, scalp sutured, and the rats returned to their home cages for recovery. Sham operated rats underwent identical surgical preparation, but did not receive the brain injury.

Ibudilast (MN166) administration

MN166 (MediciNova, San Diego, CA) is a relatively non-selective phosphodiesterase inhibitor with anti-inflammatory actions via glial cell attenuation (Mizuno et al., 2004; Rolan et al., 2009). Treated rats received a 5-day dosing regimen of once-daily MN166 injections (10 mg/kg), beginning at 30 days following LFPI. Weight was recorded prior to each dosing and treatment administered at the same time each day to maintain constant levels across a 24 hr period. Dose selection was based on prior animal pharmacology results (Ellis AL, SFN, 2008) showing MN166 to be safe and well tolerated, yielding plasma concentration-time profiles commensurate with high dose regimens in clinical development. MN166 administered via this regimen yields plasma and CNS concentrations that are linked to molecular target actions including, most potently, macrophage migration inhibitory factor (MIF) inhibition (Cho et al., 2010) and, secondarily, PDE's -4 and -10 inhibition (Gibson et al., 2006). The relevance of MIF inhibition in disorders of neuroimmune function such as neuropathic pain has recently been well demonstrated (Wang et al., 2011).

Neuromotor Tests

Baseline testing of motor, vestibular and locomotive performance in all groups was conducted immediately prior to surgery and again, at one-month following injury. These tests included ipsilateral and contralateral assessment of forelimb and hindlimb use to assess motor function, locomotion, limb use and limb preference (Bland et al., 2000; Bland et al., 2001), toe spread to assess gross motor response (Nitz et al., 1986), placing to assess visual and vestibular function (Schallert et al., 2000; Woodlee et al., 2005), catalepsy rod test to assess postural support and mobility (Sanberg et al., 1988), bracing to assess postural stability and catalepsy (Schallert et al., 1979; Morrissey et al., 1989) and air righting to assess dynamic vestibular function (Pellis et al., 1991b; Pellis et al., 1991a). Scoring ranged from 0 (severely impaired) to 5 (normal strength and function). The individual test scores were summed and a composite neuromotor score (0–45) was then generated for each animal. In addition to the composite neuromotor score, limb-use asymmetry was assessed during spontaneous exploration in the cylinder task, a common measure of motor forelimb function following central nervous system injury in rats (Schallert et al., 2000; Schallert, 2006) and post-injury locomotor activity was assessed through distance traveled on a running wheel, both tasks were scored for 5 minutes under red light (~90 lux).

Behavioral measures

A novel environment was used to assess basal anxiety levels in response to a minor stressor (Dellu et al., 1996), before a major stressor (shock) was introduced at one-month post injury. These tests were utilized to assess enhanced freezing behaviors following injury alone and exacerbated freezing in response to aversive stimuli found in our previous investigation (Rodgers et al., 2012). Shock was chosen as the major stressor because it results in freezing behavior, which is a simple reproducible response elicited as a defense reaction in both conditioned and unconditioned fearful situations (Rosen, 2004). Pathological anxiety involves exaggerated fear, characterized by hypervigilance and readiness to respond to danger or negative events (Rosen, 1998), freezing behavior is part of an anticipatory response to stress or danger.

The novel environment consisted of a standard rat cage with one vertically and one horizontally striped wall. No aversive stimuli were introduced in this context and no conditioning occurred. Rats were tested (5 minutes) and the percent of freezing behavior was assessed. Freezing was defined as the absence of movement except for heart beat/respiration, and was recorded in 10 sec intervals. Freezing behavior in the novel environment was measured after administration of a foot shock in a separate apparatus. The shock apparatus consisted of two chambers placed inside sound-attenuating chests.

The floor of each chamber consisted of 18 stainless steel rods (4 mm diameter), spaced 1.5 cm center-to-center and wired to a shock generator and scrambler (Colbourn Instruments, Allentown, PA). An automated program delivered a 2-sec/1.5 mA electric shock. Rats were transported in black buckets and shocked immediately upon entry to chambers. Following shock, rats were returned to their home cages.

Timeline for behavioral testing

Testing was performed at one-month through six-months post-injury. A single shock was delivered after neuromotor testing was completed at the one-month time point.

Immunohistochemistry

Rats were intracardially perfused with 0.9% saline and tissue was collected, then fixed with 4% paraformaldehyde overnight at 4°C. Tissue was transferred to a 30% sucrose PBS solution for 1-2 days, then stored at -80 °C. Brains were sectioned at 20 µm and mounted onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA) using a cryostat at −22 °C. Brain sections were post-fixed with 4% PFA for 15 min at room temperature, then treated with 0.3% H_2O_2 for 30 min at room temperature. Immunoreactivity in brain regions associated with anxiety (insula and amygdala) was assessed for markers of microglia (CD11b/c; OX42 labeling) and astrocytes (glial fibrillary acidic protein; GFAP), using an avidin-biotin-horseradish peroxidase (ABC) reaction (Loram et al., 2009). The sections were incubated at 4 °C overnight in either mouse anti-rat OX-42 (1:100; BD Biosciences Pharmingen, San Jose, CA) or mouse anti-pig GFAP (1:100; MP Biomedicals, Aurora, OH). The next day, sections were incubated at room temperature for 2 h with biotinylated goat anti-mouse IgG antibody (1:200; Jackson ImmunoResearch, West Grove, PA). Sections were washed and incubated for 2 h at room temperature in ABC (1:400 Vector Laboratories, Burlingame, CA) and reacted with 3', 3-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO). Sections were air-dried overnight and then dehydrated with graded alcohols, cleared in Histoclear and coverslipped with Permount (Fisher Scientific, Fairlawn, NJ).

Image Analysis

Slides were viewed with an Olympus BX-61 microscope, using Olympus Microsuite software (Olympus America, Melville, NY), with bright-field illumination at 10X magnification. Densitometric analysis was performed using Scion Image software. Images were analyzed, under blinded conditions, in NIH ImageJ using gray scale. Signal pixels of a region of interest were defined as having gray values 3.5 standard deviations above the mean gray value of a cell-poor area close to the region of interest. The number of pixels and the average gray values above the set background were then computed for each region of interest and multiplied, giving an integrated densitometric measurement. Six measurements were made for each region of interest; the measurements were then averaged to obtain a single integrated density value per rat, per region.

Statistical Analyses

Results are expressed as mean ± SEM. Analyses for behavioral measures used analysis of variance (ANOVA) with repeated measures (time after injury), and treatment as the independent variable. The integrated density was measured at one time point (six-months post-injury) and utilized one-way ANOVAs to compare regions between groups. Tukey's honestly significant difference (HSD) multiple means comparisons were used to analyze post hoc differences. Data were analyzed using SPSS® Statistics software and, in all cases, statistical significance was set at $p < 0.05$.

Results

Neuromotor composite scores of the brain-injured groups (LFPI+MN166, LFPI+vehicle) did not significantly differ from controls ($F(3, 20) = 0.383$, $p = 0.766$). Rats in all groups consistently received normal scores on forelimb and hindlimb use, toe spread, placing, catalepsy rod, bracing, and air righting tests, indicating no impairments in motor, vestibular or locomotive functioning due to TBI. There were also no significant between group differences in limb-use asymmetry observed for contralateral ($F(3, 20) = 0.058$, $p = 0.981$) and ipsilateral ($F(3, 20) = 0.285$, $p = 0.836$) forelimb use during vertical exploratory behavior in the cylinder task, indicating no limb-use bias due to injury (Figure 1A). No significant between group differences were found in locomotor performance evidenced by distance traveled during the running wheel activity ($F(3, 20) = 0.152$, $p = 0.464$), revealing no post-injury impairments in locomotion (Figure 1B).

LFPI-induced increases in freezing behavior were observed when rats were placed in a novel context following shock in a separate environment (Figure 2; $F(3, 20) = 9.029$, $p = 0.001$). Exposed only to this minor additional stressor and prior to treatment with either MN166 or vehicle, LPFI rats (Figure 2; white and black bars) froze approximately twice as long as sham-operated rats (Figure 2; light and dark gray bars) at the one-month time point: LFPI+vehicle ($p = 0.025$ vs. Sham+vehicle and $p = 0.029$ vs. Sham+MN166) and LFPI+beforeMN166 ($p = 0.029$ vs. Sham+vehicle and $p = 0.034$ vs. Sham+MN166), while the both LFPI groups did not differ ($p = 0.940$) statistically.

At two-months post-injury, following treatment with MN166 or vehicle, freezing in both sham-operated groups remained constant at approximately 25%. Freezing behavior in vehicle injected LFPI rats remained consistently higher than these controls, (p

= 0.025 vs. Sham+vehicle and p = 0.009 vs. Sham+MN166), while freezing differences between Sham+vehicle and Sham+MN166 control groups and treated LFPI+MN166 rats no longer reached significance (p = 0.486 and p = 0.257, respectively). Untreated LFPI+vehicle rats froze approximately 20% more than LFPI+MN166 rats; although this did not reach significance at the two-month time point (p = 0.100) and 30% more than sham-operated controls at the two-month post-injury measurement.

At three-months through six-months post-injury, freezing averages for Sham+MN166 and Sham+vehicle control groups again remained constant (20% and 25%, respectively). Freezing behavior in vehicle injected LFPI rats remained consistently higher than these controls at all post-treatment time points: three-month (p = 0.000 vs. Sham+vehicle and p = 0.000 vs. Sham+MN166), four-month (p = 0.102 vs. Sham+vehicle and p = 0.007 vs. Sham+MN166), five-month (p = 0.049 vs. Sham+vehicle and p = 0.013 vs. Sham+MN166), and six-month (p = 0.133 vs. Sham+vehicle and p = 0.035 vs. Sham+MN166). The behavior of treated LFPI rats remained indistinguishable from controls. Freezing differences between Sham+MN166 and Sham+vehicle injected control groups and LFPI+MN166 treated rats did not reach significance at any of the post-treatment time points: three-month (p = 0.433 vs. Sham+vehicle and p = 0.432 vs. Sham+MN166), four-month (p = 0.575 vs. Sham+vehicle and p = 0.484 vs. Sham+MN166), five-month (p = 0.680 vs. Sham+vehicle and p = 0.836 vs. Sham+MN166), and six-month (p = 0.343 vs. Sham+vehicle and p = 0.903 vs. Sham+MN166). In contrast, untreated LFPI+vehicle injected rats froze significantly more than treated rats, approximately twice that of the LFPI+MN166 treated rats across all post-treatment time points: three-month (p = 0.003), four-month ($p = 0.034$), five-month ($p = 0.021$), and six-month ($p = 0.022$).

OX-42 and GFAP immunoreactivity (reflecting microglia and astrocytic activation, respectively) was assessed in the insula and amygdala in MN166 and vehicle injected LFPI rats for comparison to sham-operated controls. Representative images (20X), showing GFAP immunoreactivity in several of these regions, are shown in Figure 3, revealing normal astrocyte morphology in both MN166 and vehicle injected sham controls. LFPI+vehicle rats showed clear signs of reactive astrocytes (Figure 3; bottom row), while LFPI rats treated with MN166 (Figure 3; third row) were difficult to differentiate from sham-operated control groups.

Immunohistochemistry revealed increased GFAP labeling in both brain regions examined, confirming that astroglial activation was significantly greater in LFPI+vehicle compared to other groups in insula (Figure 4A; left graph; $F(3, 140) = 3.761$, $p = 0.012$) and amygdala (Figure 4B; left graph; $F(3, 140) = 6.025$, $p = 0.001$). In contrast, no differences in GFAP labeling were observed between sham-operated and LFPI+MN166 groups in either region: insula ($p = 0.911$ vs. Sham+vehicle and $p = 0.611$ vs. Sham+MN166) or amygdala ($p = 0.750$ vs. Sham+vehicle and $p = 0.419$ vs. Sham+MN166). While MN166 treated LFPI rats were not distinguishable from sham-operated controls, post-hoc analyses revealed that LFPI+vehicle rats had significantly greater astrocytic activation in both brain regions as compared to controls (Figure 4A-B, left graphs): insula ($p = 0.005$ vs. LFPI+MN166, $p = 0.004$ vs. Sham+vehicle, and $p = 0.022$ vs. Sham+MN166) and amygdala ($p = 0.002$ vs. LFPI+MN166, $p = 0.005$ vs. Sham+vehicle, and $p = 0.000$ vs. Sham+MN166).

Analysis of GFAP immunoreactivity in sub-regions of the insula (Figure 4A; right graph), also revealed that LFPI+vehicle rats had increased GFAP labeling in agranular ($F(3, 140) = 2.493$, $p = 0.063$), dysgranular ($F(3, 140) = 7.388$, $p = 0.000$) and granular ($F(3, 140) = 2.998$, $p = 0.033$) insular regions. No significant differences between sham-operated and LFPI+MN166 groups were found in the sub-regions of the insula: agranular ($p = 0.739$ vs. Sham+vehicle and $p = 0.686$ vs. Sham+MN166); dysgranular ($p = 0.206$ vs. Sham+vehicle and $p = 0.186$ vs. Sham+MN166) or granular ($p = 0.153$ vs. Sham+vehicle and $p = 0.864$ vs. Sham+MN166). Untreated, LFPI+vehicle rats had greater astrocytic activation in all three sub-regions as compared to controls (Figure 4A, right graph): agranular ($p = 0.031$ vs. LFPI+MN166, $p = 0.013$ vs. Sham+vehicle, and $p = 0.079$ vs. Sham+MN166); dysgranular ($p = 0.000$ vs. LFPI+MN166, $p = 0.001$ vs. Sham+vehicle, and $p = 0.002$ vs. Sham+MN166) and granular ($p = 0.124$ vs. LFPI+MN166, $p = 0.003$ vs. Sham+vehicle, and $p = 0.087$ vs. Sham+MN166).

In the sub-regions of the amygdala (Figure 4B; right graph), GFAP labeling in LFPI+vehicle rats was significantly increased in BLA ($F(3, 140) = 39.154$, $p = 0.000$) and CE ($F(3, 140) = 12.073$, $p = 0.000$) nuclei compared to controls. Post-hoc analyses revealed that LFPI+vehicle rats had significantly greater astrocytic activation in both sub-regions: CE ($p = 0.000$ vs. LFPI+MN166, $p = 0.001$ vs. Sham+vehicle, and $p = 0.000$ vs. Sham+MN166) and BLA ($p = 0.000$ vs. LFPI+MN166, $p = 0.000$ vs. Sham+vehicle, and $p = 0.000$ vs. Sham+MN166). MN166 treated LFPI rats had significantly less GFAP expression than Sham+vehicle controls in CE ($p = 0.031$), but did not differ from Sham+MN166 treated rats ($p = 0.583$). LFPI+MN166 treated rats also did not differ from sham controls in the BLA ($p = 0.575$ vs. Sham+vehicle and $p = 0.063$ vs. Sham+MN166).

LFPI+vehicle rats also showed significantly increased microglial activation, as measured by OX-42 labeling, compared to control groups (Figure 4C), but this was restricted to sub-regions of the amygdala: CE $F(3, 140) = 9.290$, $p = 0.000$), and also approached significance in BLA $F(3, 140) = 2.399$, $p = 0.071$) nuclei. Post-hoc analysis revealed significant increases in microglial activation for LFPI+vehicle rats in CE ($p = 0.000$ vs. LFPI+MN166, $p = 0.000$ vs. Sham+vehicle, and $p = 0.000$ vs. Sham+MN166) and BLA ($p = 0.009$ vs. Sham+MN166). No differences in OX-42 labeling were observed between sham-operated and LFPI+MN166 groups in amygdala, nor were any significant between group differences found in OX-42 expression for the insula.

Discussion

LFPI-induced anxiety-like behaviors are found at long-term, post-injury time points in untreated brain-injured rats, as compared to those treated with MN166. Pharmacological suppression of immune responses at one-month post-injury, when anxiety-like behavior has fully developed, markedly reduces long-term behavioral and immunological impairments following TBI (out to six-months) and restores MN166 treated rats to levels indistinguishable from sham-operated controls. These findings not only implicate chronic neuroinflammation in the development of anxiety-like behaviors following TBI, but also show that delayed treatment is capable of reversing established post-traumatic anxiety behaviors. To our knowledge, this is the first study to examine delayed immunosuppression at long-term injury time points, as other immunosuppressive treatments targeting anxiety-like behaviors have been administered prior to or within hours of injury (Homsi et al., 2009; Homsi et al., 2010; Kovesdi et al., 2012; Lee et al., 2012; Lopez et al., 2012; Rodgers et al., 2012; Siopi et al., 2012). These results indicate that the persistence of post-traumatic anxiety may reflect chronic neuroinflammatory neuropathy, a possibility supported by observation of activated microglia and astrocytes, key cells mediating inflammatory processes, many years following injury in long-term survivors of TBI (Gentleman et al., 2004; Streit et al., 2004; Nagamoto-Combs et al., 2007; Ramlackhansingh et al., 2011).

Chronic post-traumatic neuroinflammation suggests the presence of a self-perpetuating positive feedback loop, possibly involving reactivation and further promotion of inflammatory mediators following injury in an inflammation-damage-inflammation cycle (Namas et al., 2009). Stressed, damaged, and injured cells release endogenous danger signals, which trigger local inflammatory responses needed for tissue repair and regeneration (Gallucci and Matzinger, 2001; Oppenheim and Yang, 2005; Oppenheim et al., 2007; Hirsiger et al., 2012). Damage-associated molecular patterns (DAMPs) play an important role in the propagation of the proinflammatory cascade of innate immunity, promoting the release of cytokines and other inflammatory mediators (Bianchi, 2007; Namas et al., 2009). DAMPs initiate the innate immune response through the activation of antigen presenting cells (APCs) which up-regulate co-stimulatory and major histocompatibility complex (MHC) molecules (Gallucci and Matzinger, 2001; Matzinger, 2002; Hirsiger et al., 2012). APCs respond to endogenous signals through toll-like receptors (TLRs), which recognize a variety of DAMPs and act as pattern recognition receptors (PRRs) for endogenous molecules.

Microglia are the resident immunological cells and primary APCs of the CNS, remaining quiescent until activated through TLR engagement with DAMPs to perform effector inflammatory and APC functions (Olson and Miller, 2004). Microglial cells contribute to neuroinflammation in response to DAMPS by secreting proinflammatory cytokines such as IL-1 and TNF-α, which amplify the inflammatory response by initiating the production of other cytokines and promoting microglial proliferation and activation of astrocytes (Namas et al., 2009). This so called 'cytokine cycle' may initiate a prolonged and self-perpetuating inflammatory response in the brain that exceeds early neuroprotection and results in neurodegenerative changes (Griffin et al., 1998; Gentleman et al., 2004). Sustained glial responses have been shown to result in secondary tissue damage (Gasque et al., 2000; Simi et al., 2007; Hailer, 2008; Lehnardt, 2010) and neuronal death (Sternberg, 1997; Brown and Bal-Price, 2003; Schmidt et al., 2005; Beattie et al., 2010) capable of continuing the inflammatory cycle.

Chronic inflammation has been seen in a number of studies examining patients with trauma-related anxiety disorders, reporting peripheral elevations of TNF-α, IFNγ, IL-1β and IL-6 in patients with PTSD (Spivak et al., 1997; Rohleder et al., 2004; Tucker et al., 2004; von Kanel et al., 2007), elevations of TNF-α and IL-6 in patients with OCD (Konuk et al., 2007), and elevations in proinflammatory cytokines and chemokines (MCP-1, MIP-1α. IL-1α, IL-1β, IL-6, IL-8, Eotaxin, GM-CSF and IFNγ) in individuals with panic disorder (PD) and PTSD (Hoge et al., 2009). Despite compelling evidence implicating excessive inflammatory actions and a generalized inflammatory state in the development of anxiety disorders following TBI, central measures of proinflammatory cytokine elevations specifically related to human PTSD and other anxiety disorders have not yet been performed. However, the current results provide clear evidence for chronic neuroinflammation in the development and maintenance of post-traumatic anxiety in an animal model, as indicated by elevated astroglial and microglial immunoreactivity in the amygdala and insula at six-months post-injury.

The amygdala and insula have long been associated with human anxiety disorders. Studies in patients with PTSD implicate exaggerated responses of the amygdala and insula (Rauch et al., 1997; Simmons et al., 2006; Stein et al., 2007; Shin and Liberzon, 2010; Carlson et al., 2011), impaired inhibition of medial prefrontal cortex and anterior cingulate (Davidson, 2002; Shin et al., 2006; Milad et al., 2009; Shin and Liberzon, 2010) and decreased hippocampal volume (Bremner et al., 1995; Sapolsky, 2000; Shin et al., 2006). Other neuroimaging reports of patients with non-trauma-related obsessive/compulsive disorder and phobia, as well as those with PTSD, have revealed that aversive anticipation (a hallmark of anxiety) involves increased activation of both the amygdala and insula (Simmons et al., 2006). Evidence for the specific involvement of these brain areas in human post-traumatic anxiety is complemented by animal models, including findings of increased PTSD-related traits and increased Stathmin 1 (a protein known to increase fear responses) expression in the amygdala following blast injury (Elder et al., 2012); increased fear conditioning and up-regulation of excitatory N-methyl-D-aspartate receptors (crucial receptors for normal fear learning and memory) in the amygdala following concussive injury (Reger et al., 2012); and our current results showing increased anxiety-like behavior and reactive gliosis in insula and amygdala at long-term time points following LFPI.

While the exact role of the immune system in the pathogenesis of anxiety disorders following TBI remains unknown, neuroinflammation is emerging as a potential target. The present findings of treatment related reductions in anxiety-like behaviors and reactive gliosis in brain regions associated with anxiety, supports the use of immunosuppression to improve functional outcome following TBI. Peri-injury and immediate post-injury immunosuppression have been found to be neuroprotective following TBI in rodents, resulting in increased structural preservation and improved functional outcomes (Hailer, 2008). Early administration of the immunosuppressant drugs minocycline, statins, cyclosporin A and FK506 have been shown to exert anti-inflammatory effects through the suppression of microglial and astroglial production of IL-1β, TNF-α and IL-6, resulting in reduced functional deficits, cerebral edema and brain lesion volumes (Chen et al., 2007; Homsi et al., 2009; Li et al., 2009; Homsi et al., 2010; Siopi et al., 2012), improving mitochondrial preservation, reducing dendritic spine loss, and improving cognitive performance and functional motor recovery (Alessandri et al., 2002; Campbell et al., 2011). Our previous investigation found that peri-injury Ibudilast treatment attenuated glial cell activation at the time of injury, resulting in reduced anxiety-like behaviors and immunological impairments following LFPI (Rodgers et al., 2012).

These immunosuppressant drugs all have direct inhibitory effects on microglia and astrocytes, leading to better functional recovery following TBI; however, these treatments require rapid administration and reduce the therapeutic window to the day of injury. The current work shows reversal of established anxiety-like behaviors and reactive gliosis at one-month post injury, delayed treatment time points that have not been tested with any other immunosuppressive interventions in spite of substantial evidence that many molecular, biochemical, and immunological changes occur for months following injury and that clinical intervention may not be possible at early stages of TBI.

Our finding that delayed immunosuppression is capable of reversing established post-traumatic anxiety behaviors and immunological impairments through six-months following TBI contributes to growing evidence that the critical window for treatment following TBI can be expanded to include those suffering from long-term TBI-related disabilities. Studies have shown that delayed treatment (24 hr) with erythropoietin (EPO), a novel neuroprotective cytokine found to improve neuronal survival through the attenuation of cytokine production and inflammation, improved sensorimotor functional recovery, reduced hippocampal cell loss, enhanced neurogenesis, and improved neurological outcomes following controlled cortical impact and weight drop rodent TBI models (Yatsiv et al., 2005; Xiong et al., 2010). Similarly, a recent study reported reduced chronic inflammation and neurodegeneration after activation of metabotropic glutamate receptor 5 (mGluR5) with the specific agonist (RS)-2-chloro-5-hydroxyphenylglycine (CHPG), which has been shown to decrease microglial activation and release of associated proinflammatory mediators. The study delayed treatment until one-month following controlled cortical impact in mice, delivering a single intracerebroventricular (icv) injection of CHPG, and the results revealed reductions in reactive gliosis, hippocampal cell loss, reduced lesion progression, and improved motor and cognitive recovery compared to untreated controls (Byrnes et al., 2012).

Immunosuppression of chronic neuroinflammation may hold promise as a therapeutic target in the treatment of established anxiety disorders following TBI. It has been shown here that inflammation produced by neuroimmune responses following injury play a role in TBI-induced anxiety, and delayed immunosuppression leads to better functional outcomes at long-term post-injury treatment points following TBI. Delayed, post-injury suppression of glial cell activation could therefore expand the clinical window for treatment of TBI-induced anxiety disorders in humans.

References

Alessandri B, Rice AC, Levasseur J, DeFord M, Hamm RJ, Bullock MR (2002) Cyclosporin A improves brain tissue oxygen consumption and learning/memory performance after lateral fluid percussion injury in rats. Journal of neurotrauma 19:829-841.

Beattie MS, Ferguson AR, Bresnahan JC (2010) AMPA-receptor trafficking and injury-induced cell death. Eur J Neurosci 32:290-297.

Bianchi ME (2007) DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol 81:1-5.

Bland ST, Pillai RN, Aronowski J, Grotta JC, Schallert T (2001) Early overuse and disuse of the affected forelimb after moderately severe intraluminal suture occlusion of the middle cerebral artery in rats. Behav Brain Res 126:33-41.

Bland ST, Schallert T, Strong R, Aronowski J, Grotta JC, Feeney DM (2000) Early exclusive use of the affected forelimb after moderate transient focal ischemia in rats : functional and anatomic outcome. Stroke 31:1144-1152.

Bremner JD, Randall P, Scott TM, Bronen RA, Seibyl JP, Southwick SM, Delaney RC, McCarthy G, Charney DS, Innis RB (1995) MRI-based measurement of hippocampal volume in patients with combat-related posttraumatic stress disorder. Am J Psychiatry 152:973-981.

Brown GC, Bal-Price A (2003) Inflammatory neurodegeneration mediated by nitric oxide, glutamate, and mitochondria. Mol Neurobiol 27:325-355.

Byrnes KR, Loane DJ, Stoica BA, Zhang J, Faden AI (2012) Delayed mGluR5 activation limits neuroinflammation and neurodegeneration after traumatic brain injury. J Neuroinflammation 9:43.

Campbell JN, Churn SB, Register D (2011) Traumatic Brain Injury Causes an FK506-Sensitive Loss and an Overgrowth of Dendritic Spines in Rat Forebrain. Journal of neurotrauma.

Carlson JM, Greenberg T, Rubin D, Mujica-Parodi LR (2011) Feeling anxious: anticipatory amygdalo-insular response predicts the feeling of anxious anticipation. Soc Cogn Affect Neurosci 6:74-81.

Chen SF, Hung TH, Chen CC, Lin KH, Huang YN, Tsai HC, Wang JY (2007) Lovastatin improves histological and functional outcomes and reduces inflammation after experimental traumatic brain injury. Life Sci 81:288-298.

Cho Y, Crichlow GV, Vermeire JJ, Leng L, Du X, Hodsdon ME, Bucala R, Cappello M, Gross M, Gaeta F, Johnson K, Lolis EJ (2010) Allosteric inhibition of macrophage migration inhibitory factor revealed by ibudilast. Proceedings of the National Academy of Sciences of the United States of America 107:11313-11318.

Connor TJ, Song C, Leonard BE, Merali Z, Anisman H (1998) An assessment of the effects of central interleukin-1beta, -2, -6, and tumor necrosis factor-alpha administration on some behavioural, neurochemical, endocrine and immune parameters in the rat. Neuroscience 84:923-933.

Cragnolini AB, Schioth HB, Scimonelli TN (2006) Anxiety-like behavior induced by IL-1beta is modulated by alpha-MSH through central melanocortin-4 receptors. Peptides 27:1451-1456.

Davidson RJ (2002) Anxiety and affective style: role of prefrontal cortex and amygdala. Biological psychiatry 51:68-80.

Deb S, Lyons I, Koutzoukis C, Ali I, McCarthy G (1999) Rate of psychiatric illness 1 year after traumatic brain injury. Am J Psychiatry 156:374-378.

Dellu F, Mayo W, Vallee M, Maccari S, Piazza PV, Le Moal M, Simon H (1996) Behavioral reactivity to novelty during youth as a predictive factor of stress-induced corticosterone secretion in the elderly--a life-span study in rats. Psychoneuroendocrinology 21:441-453.

Elder GA, Dorr NP, De Gasperi R, Gama Sosa MA, Shaughness MC, Maudlin-Jeronimo E, Hall AA, McCarron RM, Ahlers ST (2012) Blast exposure induces post-traumatic stress disorder-related traits in a rat model of mild traumatic brain injury. Journal of neurotrauma 29:2564-2575.

Ellis AL WJ, Brown K, Blackwood C, Ramos K, Starnes C, Maier SF, and Watkins LR (SFN, 2008) Characterization of exaggerated pain behavior and glial activation in a novel rat model of spinal cord injury. In.

Faul M, Xu, L., Wald, M.M., Coronado, V.G. (2010) Traumatic Brain Injury in the United States: Emergency Department Visits, Hospitalizations and Deaths 2002–2006. 1-74.

Frey LC, Hellier J, Unkart C, Lepkin A, Howard A, Hasebroock K, Serkova N, Liang L, Patel M, Soltesz I, Staley K (2009) A novel apparatus for lateral fluid percussion injury in the rat. J Neurosci Methods 177:267-272.

Gallucci S, Matzinger P (2001) Danger signals: SOS to the immune system. Curr Opin Immunol 13:114-119.

Gasque P, Dean YD, McGreal EP, VanBeek J, Morgan BP (2000) Complement components of the innate immune system in health and disease in the CNS. Immunopharmacology 49:171-186.

Gehrmann J, Banati RB, Kreutzberg GW (1993) Microglia in the immune surveillance of the brain: human microglia constitutively express HLA-DR molecules. J Neuroimmunol 48:189-198.

Gehrmann J, Matsumoto Y, Kreutzberg GW (1995) Microglia: intrinsic immuneffector cell of the brain. Brain research Brain research reviews 20:269-287.

Gentleman SM, Leclercq PD, Moyes L, Graham DI, Smith C, Griffin WS, Nicoll JA (2004) Long-term intracerebral inflammatory response after traumatic brain injury. Forensic Sci Int 146:97-104.

Gibson LC, Hastings SF, McPhee I, Clayton RA, Darroch CE, Mackenzie A, Mackenzie FL, Nagasawa M, Stevens PA, Mackenzie SJ (2006) The inhibitory profile of Ibudilast against the human phosphodiesterase enzyme family. European journal of pharmacology 538:39-42.

Griffin WS, Sheng JG, Royston MC, Gentleman SM, McKenzie JE, Graham DI, Roberts GW, Mrak RE (1998) Glial-neuronal interactions in Alzheimer's disease: the potential role of a 'cytokine cycle' in disease progression. Brain Pathol 8:65-72.

Hailer NP (2008) Immunosuppression after traumatic or ischemic CNS damage: it is neuroprotective and illuminates the role of microglial cells. Prog Neurobiol 84:211-233.

Hiott DW, Labbate L (2002) Anxiety disorders associated with traumatic brain injuries. NeuroRehabilitation 17:345-355.

Hirsiger S, Simmen HP, Werner CM, Wanner GA, Rittirsch D (2012) Danger signals activating the immune response after trauma. Mediators Inflamm 2012:315941.

Hoge EA, Brandstetter K, Moshier S, Pollack MH, Wong KK, Simon NM (2009) Broad spectrum of cytokine abnormalities in panic disorder and posttraumatic stress disorder. Depress Anxiety 26:447-455.

Homsi S, Federico F, Croci N, Palmier B, Plotkine M, Marchand-Leroux C, Jafarian-Tehrani M (2009) Minocycline effects on cerebral edema: relations with inflammatory and oxidative stress markers following traumatic brain injury in mice. Brain research 1291:122-132.

Homsi S, Piaggio T, Croci N, Noble F, Plotkine M, Marchand-Leroux C, Jafarian-Tehrani M (2010) Blockade of acute microglial activation by minocycline promotes neuroprotection and reduces locomotor hyperactivity after closed head injury in mice: a twelve-week follow-up study. Journal of neurotrauma 27:911-921.

Konuk N, Tekin IO, Ozturk U, Atik L, Atasoy N, Bektas S, Erdogan A (2007) Plasma levels of tumor necrosis factor-alpha and interleukin-6 in obsessive compulsive disorder. Mediators Inflamm 2007:65704.

Koponen S, Taiminen T, Portin R, Himanen L, Isoniemi H, Heinonen H, Hinkka S, Tenovuo O (2002) Axis I and II psychiatric disorders after traumatic brain injury: a 30-year follow-up study. Am J Psychiatry 159:1315-1321.

Kovesdi E, Kamnaksh A, Wingo D, Ahmed F, Grunberg NE, Long JB, Kasper CE, Agoston DV (2012) Acute Minocycline Treatment Mitigates the Symptoms of Mild Blast-Induced Traumatic Brain Injury. Front Neurol 3:111.

Lee HF, Lee TS, Kou YR (2012) Anti-inflammatory and neuroprotective effects of triptolide on traumatic brain injury in rats. Respir Physiol Neurobiol 182:1-8.

Lehnardt S (2010) Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. Glia 58:253-263.

Li B, Mahmood A, Lu D, Wu H, Xiong Y, Qu C, Chopp M (2009) Simvastatin attenuates microglial cells and astrocyte activation and decreases interleukin-1beta level after traumatic brain injury. Neurosurgery 65:179-185; discussion 185-176.

Lopez NE, Gaston L, Lopez KR, Coimbra RC, Hageny A, Putnam J, Eliceiri B, Coimbra R, Bansal V (2012) Early ghrelin treatment attenuates disruption of the blood brain barrier and apoptosis after traumatic brain injury through a UCP-2 mechanism. Brain research 1489:140-148.

Loram LC, Harrison JA, Sloane EM, Hutchinson MR, Sholar P, Taylor FR, Berkelhammer D, Coats BD, Poole S, Milligan ED, Maier SF, Rieger J, Watkins LR (2009) Enduring reversal of neuropathic pain by a single intrathecal injection of adenosine 2A receptor agonists: a novel therapy for neuropathic pain. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:14015-14025.

Matzinger P (1998) An innate sense of danger. Semin Immunol 10:399-415.

Matzinger P (2002) An innate sense of danger. Annals of the New York Academy of Sciences 961:341-342.

McIntosh TK, Vink R, Noble L, Yamakami I, Fernyak S, Soares H, Faden AL (1989) Traumatic brain injury in the rat: characterization of a lateral fluid-percussion model. Neuroscience 28:233-244.

Milad MR, Pitman RK, Ellis CB, Gold AL, Shin LM, Lasko NB, Zeidan MA, Handwerger K, Orr SP, Rauch SL (2009) Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. Biological psychiatry 66:1075-1082.

Mizuno T, Kurotani T, Komatsu Y, Kawanokuchi J, Kato H, Mitsuma N, Suzumura A (2004) Neuroprotective role of phosphodiesterase inhibitor ibudilast on neuronal cell death induced by activated microglia. Neuropharmacology 46:404-411.

Morrissey TK, Pellis SM, Pellis VC, Teitelbaum P (1989) Seemingly paradoxical jumping in cataleptic haloperidol-treated rats is triggered by postural instability. Behav Brain Res 35:195-207.

Morton MV, Wehman P (1995) Psychosocial and emotional sequelae of individuals with traumatic brain injury: a literature review and recommendations. Brain injury : [BI] 9:81-92.

Nagamoto-Combs K, McNeal DW, Morecraft RJ, Combs CK (2007) Prolonged microgliosis in the rhesus monkey central nervous system after traumatic brain injury. Journal of neurotrauma 24:1719-1742.

Namas R, Ghuma A, Hermus L, Zamora R, Okonkwo DO, Billiar TR, Vodovotz Y (2009) The acute inflammatory response in trauma / hemorrhage and traumatic brain injury: current state and emerging prospects. Libyan J Med 4:97-103.

Nitz AJ, Dobner JJ, Matulionis DH (1986) Pneumatic tourniquet application and nerve integrity: motor function and electrophysiology. Exp Neurol 94:264-279.

Olson JK, Miller SD (2004) Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J Immunol 173:3916-3924.

Oppenheim JJ, Yang D (2005) Alarmins: chemotactic activators of immune responses. Curr Opin Immunol 17:359-365.

Oppenheim JJ, Tewary P, de la Rosa G, Yang D (2007) Alarmins initiate host defense. Adv Exp Med Biol 601:185-194.

Pellis SM, Whishaw IQ, Pellis VC (1991a) Visual modulation of vestibularly-triggered air-righting in rats involves the superior colliculus. Behav Brain Res 46:151-156.

Pellis SM, Pellis VC, Teitelbaum P (1991b) Air righting without the cervical righting reflex in adult rats. Behav Brain Res 45:185-188.

Pugin J (2012) How tissue injury alarms the immune system and causes a systemic inflammatory response syndrome. Ann Intensive Care 2:27.

Raison CL, Miller AH (2003) When not enough is too much: the role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders. Am J Psychiatry 160:1554-1565.

Ramlackhansingh AF, Brooks DJ, Greenwood RJ, Bose SK, Turkheimer FE, Kinnunen KM, Gentleman S, Heckemann RA, Gunanayagam K, Gelosa G, Sharp DJ (2011) Inflammation after trauma: microglial activation and traumatic brain injury. Ann Neurol 70:374-383.

Rao V, Lyketsos C (2000) Neuropsychiatric sequelae of traumatic brain injury. Psychosomatics 41:95-103.

Rauch SL, Savage CR, Alpert NM, Fischman AJ, Jenike MA (1997) The functional neuroanatomy of anxiety: a study of three disorders using positron emission tomography and symptom provocation. Biological psychiatry 42:446-452.

Reger ML, Poulos AM, Buen F, Giza CC, Hovda DA, Fanselow MS (2012) Concussive brain injury enhances fear learning and excitatory processes in the amygdala. Biological psychiatry 71:335-343.

Rodgers KM, Bercum FM, McCallum DL, Rudy JW, Frey LC, Johnson KW, Watkins LR, Barth DS (2012) Acute neuroimmune modulation attenuates the development of anxiety-like freezing behavior in an animal model of traumatic brain injury. Journal of neurotrauma 29:1886-1897.

Rohleder N, Joksimovic L, Wolf JM, Kirschbaum C (2004) Hypocortisolism and increased glucocorticoid sensitivity of pro-Inflammatory cytokine production in Bosnian war refugees with posttraumatic stress disorder. Biological psychiatry 55:745-751.

Rolan P, Hutchinson M, Johnson K (2009) Ibudilast: a review of its pharmacology, efficacy and safety in respiratory and neurological disease. Expert Opin Pharmacother 10:2897-2904.

Sanberg PR, Bunsey MD, Giordano M, Norman AB (1988) The catalepsy test: its ups and downs. Behav Neurosci 102:748-759.

Sapolsky RM (2000) Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Arch Gen Psychiatry 57:925-935.

Schallert T (2006) Behavioral tests for preclinical intervention assessment. NeuroRx 3:497-504.

Schallert T, De Ryck M, Whishaw IQ, Ramirez VD, Teitelbaum P (1979) Excessive bracing reactions and their control by atropine and L-DOPA in an animal analog of Parkinsonism. Exp Neurol 64:33-43.

Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST (2000) CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. Neuropharmacology 39:777-787.

Schmidt OI, Heyde CE, Ertel W, Stahel PF (2005) Closed head injury--an inflammatory disease? Brain research Brain research reviews 48:388-399.

Shin LM, Liberzon I (2010) The neurocircuitry of fear, stress, and anxiety disorders. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 35:169-191.

Shin LM, Rauch SL, Pitman RK (2006) Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. Annals of the New York Academy of Sciences 1071:67-79.

Simi A, Tsakiri N, Wang P, Rothwell NJ (2007) Interleukin-1 and inflammatory neurodegeneration. Biochem Soc Trans 35:1122-1126.

Simmons A, Strigo I, Matthews SC, Paulus MP, Stein MB (2006) Anticipation of aversive visual stimuli is associated with increased insula activation in anxiety-prone subjects. Biological psychiatry 60:402-409.

Siopi E, Llufriu-Daben G, Fanucchi F, Plotkine M, Marchand-Leroux C, Jafarian-Tehrani M (2012) Evaluation of late cognitive impairment and anxiety states following traumatic brain injury in mice: the effect of minocycline. Neurosci Lett 511:110-115.

Sokolova ES, Lyudyno VI, Simbirtsev AS, Klimenko VM (2007) The psychomodulatory action of subpyrogenic doses of interleukin-1beta in conditions of chronic administration to rats. Neurosci Behav Physiol 37:499-504.

Spivak B, Shohat B, Mester R, Avraham S, Gil-Ad I, Bleich A, Valevski A, Weizman A (1997) Elevated levels of serum interleukin-1 beta in combat-related posttraumatic stress disorder. Biol Psychiatry 42:345-348.

Stein MB, Simmons AN, Feinstein JS, Paulus MP (2007) Increased amygdala and insula activation during emotion processing in anxiety-prone subjects. Am J Psychiatry 164:318-327.

Sternberg EM (1997) Neural-immune interactions in health and disease. J Clin Invest 100:2641-2647.

Streit WJ, Mrak RE, Griffin WS (2004) Microglia and neuroinflammation: a pathological perspective. J Neuroinflammation 1:14.

Szelenyi J, Vizi ES (2007) The catecholamine cytokine balance: interaction between the brain and the immune system. Annals of the New York Academy of Sciences 1113:311-324.

Thompson HJ, Lifshitz J, Marklund N, Grady MS, Graham DI, Hovda DA, McIntosh TK (2005) Lateral fluid percussion brain injury: a 15-year review and evaluation. Journal of neurotrauma 22:42-75.

Tucker P, Ruwe WD, Masters B, Parker DE, Hossain A, Trautman RP, Wyatt DB (2004) Neuroimmune and cortisol changes in selective serotonin reuptake inhibitor and placebo treatment of chronic posttraumatic stress disorder. Biol Psychiatry 56:121-128.

Vaishnavi S, Rao V, Fann JR (2009) Neuropsychiatric problems after traumatic brain injury: unraveling the silent epidemic. Psychosomatics 50:198-205.

von Kanel R, Hepp U, Kraemer B, Traber R, Keel M, Mica L, Schnyder U (2007) Evidence for low-grade systemic proinflammatory activity in patients with posttraumatic stress disorder. J Psychiatr Res 41:744-752.

Wang F, Xu S, Shen X, Guo X, Peng Y, Yang J (2011) Spinal macrophage migration inhibitory factor is a major contributor to rodent neuropathic pain-like hypersensitivity. Anesthesiology 114:643-659.

Woodlee MT, Asseo-Garcia AM, Zhao X, Liu SJ, Jones TA, Schallert T (2005) Testing forelimb placing "across the midline" reveals distinct, lesion-dependent patterns of recovery in rats. Exp Neurol 191:310-317.

Xiong Y, Mahmood A, Meng Y, Zhang Y, Qu C, Schallert T, Chopp M (2010) Delayed administration of erythropoietin reducing hippocampal cell loss, enhancing angiogenesis and neurogenesis, and improving functional outcome following traumatic brain injury in rats: comparison of treatment with single and triple dose. J Neurosurg 113:598-608.

Yatsiv I, Grigoriadis N, Simeonidou C, Stahel PF, Schmidt OI, Alexandrovitch AG, Tsenter J, Shohami E (2005) Erythropoietin is neuroprotective, improves functional recovery, and reduces neuronal apoptosis and inflammation in a rodent model of experimental closed head injury. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 19:1701-1703.

Zubareva OE, Klimenko VM (2009) Long-term disorders of behavior in rats induced by administration of tumor necrosis factor during early postnatal ontogenesis. Neurosci Behav Physiol 39:21-24.

Legends

Figure 1. Cylinder task and running wheel activity at one-month post-injury. (A) LFPI rats mean number of spontaneous forelimb placements (ipsilateral and contralateral) during exploratory activity in the cylinder test did not differ from controls at one-month post-injury. (B) LFPI rats mean change in distance traveled in the running wheel activity did not significantly differ from controls at one-month post-injury. Data represent mean ± SEM.

Figure 2. Freezing behavior in novel context. Sham-operated rats froze approximately 25% before treatment with MN166 or vehicle, while LFPI rats froze at significantly higher rates (~60%). Following treatment, LFPI+MN166 rats freezing behavior was reduced to (~25%) compared to LFPI+vehicle rats (~50%). This effect was significant at three-months and remained through six-months post-injury. Freezing in Sham+MN166 and Sham+vehicle rats could not be distinguished from LFPI+MN166 treated rats at all time points following treatment; while LFPI+vehicle injected rats froze significantly more than both sham groups at all post-treatment time points with the exception of the sham vehicle group at the four-month and six-month time points. Data represent mean ± SEM.

Figure 3. Representative photomicrographs (20X) depicting GFAP immunoreactivity assessed in the insula and amygdala at six-months post-injury. Vehicle-injected LFPI rats showed clear signs of reactive astrocytes (bottom row), while astrocytes from sham-operated rat tissue appear to have normal morphology (top rows). LFPI rats treated with

MN166 (third row) were difficult to differentiate from sham-operated groups. Rhinal fissure (rf) and commissural stria terminalis (cst).

Figure 4. Astroglial and microglial activation in insula and amygdala at six-months post-injury. (A-B) LFPI+vehicle rats had significantly increased in GFAP labeling in both regions, indicating higher astroglial activation compared to sham-operated and LFPI+MN166 treated rats. (C) In the CE, microglial activation was greater in LFPI+vehicle injected rats compared to both sham operated groups and LFPI+MN166 treated rats, and was approaching significance in BLA. Central amagdala (CE) and basolateral amygdala (BLA). Data represent mean± SEM.

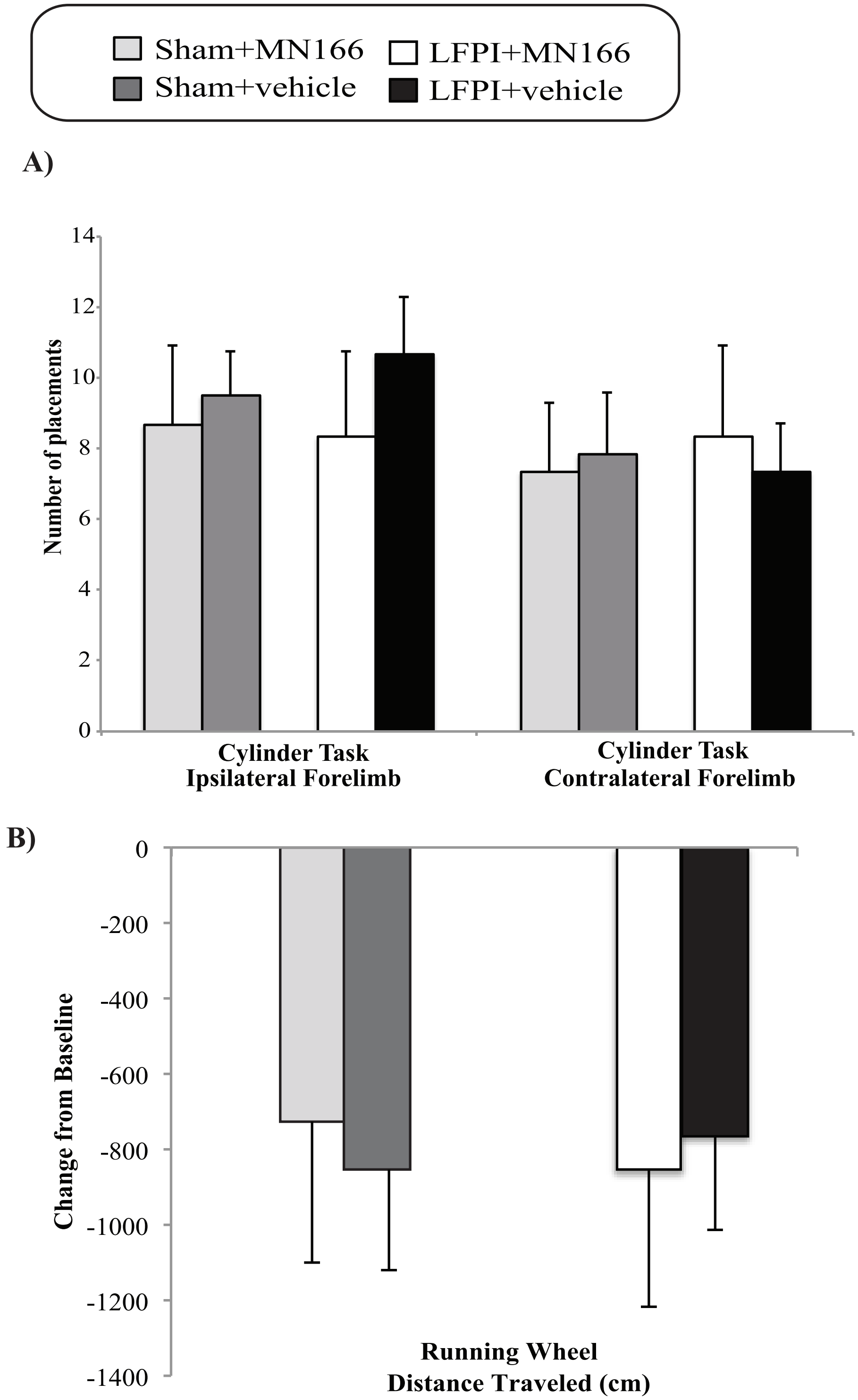
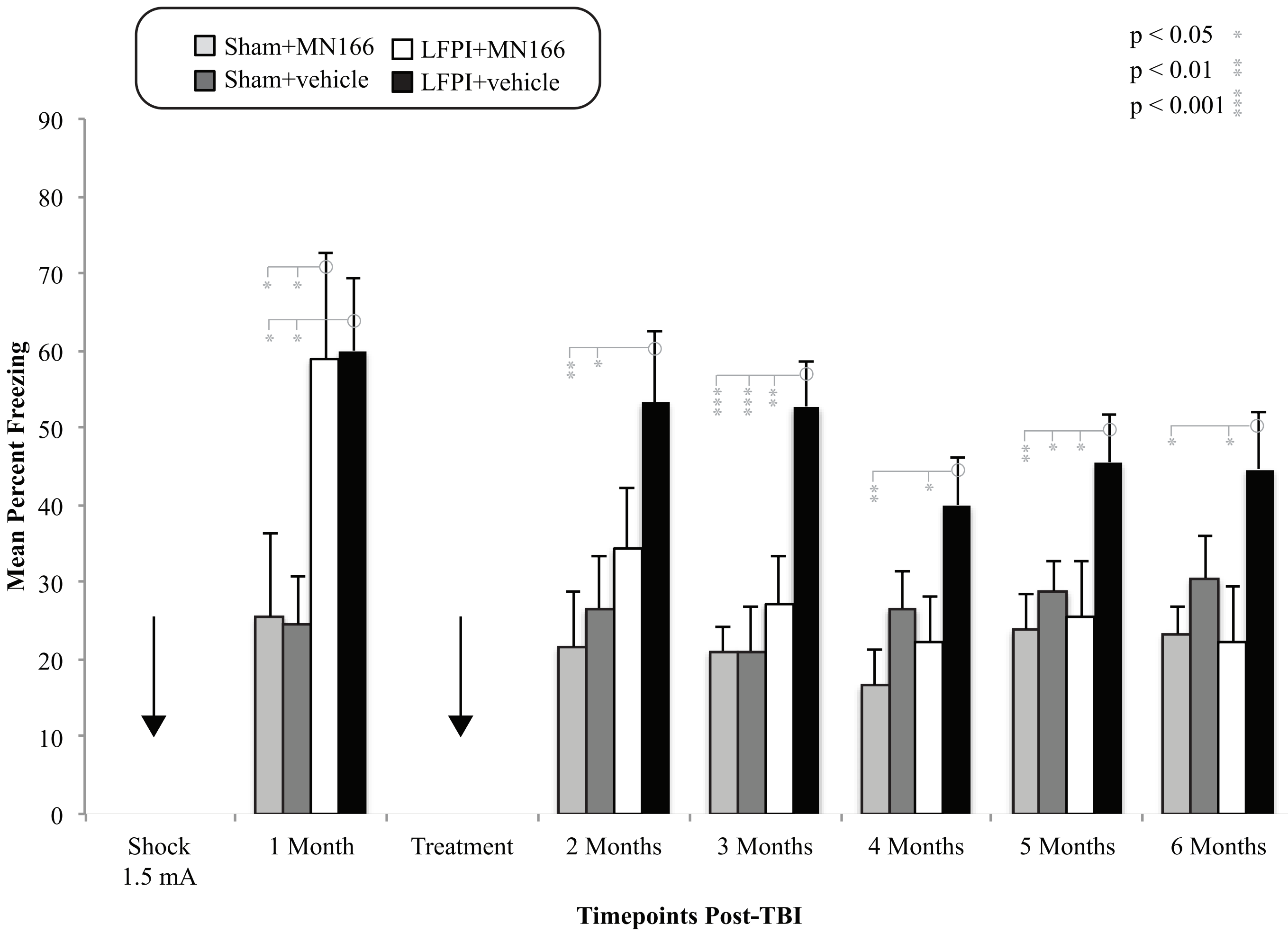

Sham+MN166
LFPI+MN166
Sham+vehicle
LFPI+vehicle
A)
Number of placements
14
12
10
8
6
4
2
0
Cylinder Task
Ipsilateral Forelimb
Cylinder Task
Contralateral Forelimb
B)
Change from Baseline
0
-200
-400
-600
-800
-1000
-1200
-1400
Running Wheel
Distance Traveled (cm)

Figure 2



Sham+MN166
LFPI+MN166
Sham+vehicle
LFPI+vehicle
p < 0.05
p < 0.01
p < 0.001
Mean Percent Freezing
90
80
70
60
50
40
30
20
10
0
Shock
1.5 mA
1 Month
Treatment
2 Months
3 Months
4 Months
5 Months
6 Months
Timepoints Post-TBI

Figure 3

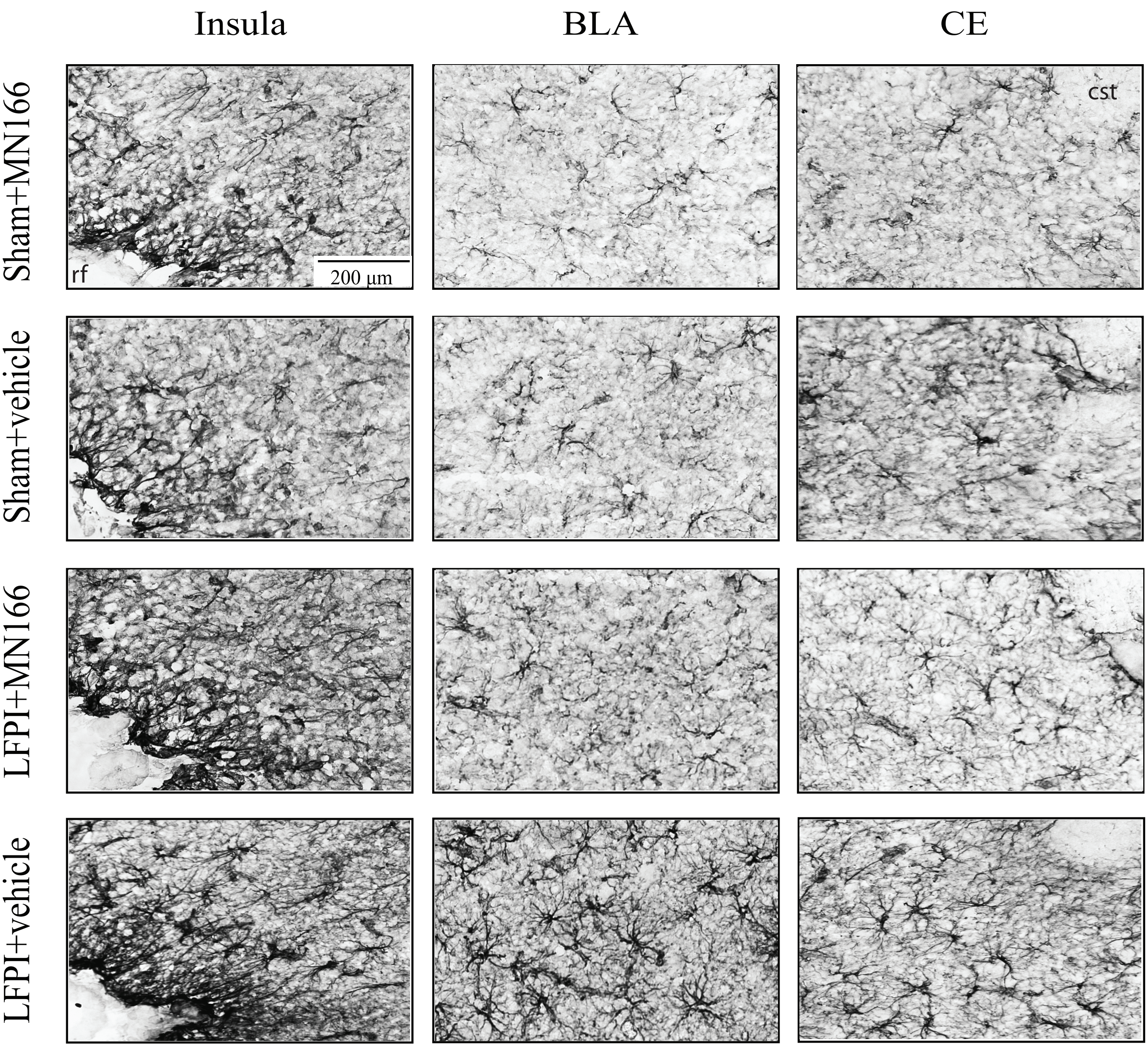
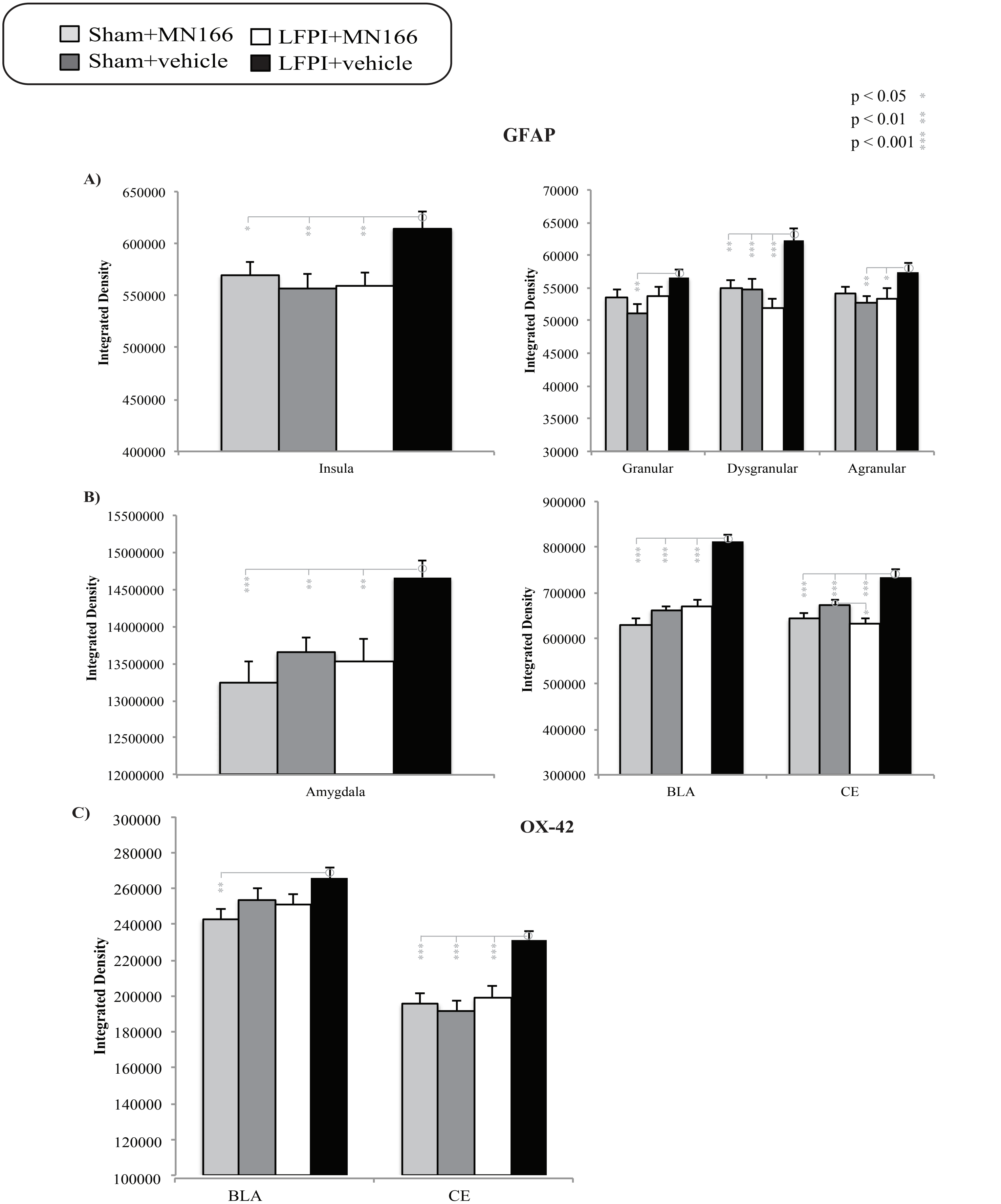

Insula
BLA
CE
Sham+MN166
Sham+vehicle
LFPI+MN166
LFPI+vehicle
rf
200 μm
cst

Figure 4



Sham+MN166
LFPI+MN166
Sham+vehicle
LFPI+vehicle
p < 0.05 *
p < 0.01 **
p < 0.001 ***
GFAP
A)
Integrated Density
650000
600000
550000
500000
450000
400000
Insula
70000
65000
60000
55000
50000
45000
40000
35000
30000
Granular
Dysgranular
Agranular
B)
15500000
15000000
14500000
14000000
13500000
13000000
12500000
12000000
Amygdala
900000
800000
700000
600000
500000
400000
300000
BLA
CE
C)
OX-42
300000
280000
260000
240000
220000
200000
180000
160000
140000
120000
100000
BLA
CE